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(54) Title: METHODS AND COMPOSITIONS FOR REGULATING T CELL SUBSETS BY MODULATING TRANSCRIPTION FACTOR ACTIVITY

(57) Abstract

Methods for modulating production of a T helper type 2 (Th2)-associated cytokine, in particular interleukin-4, by modulating the activity of one or more transcription factors that cooperate with NF-AT family proteins to regulate expression of a Th2-associated cytokine gene are disclosed. In one embodiment, the activity of a maf family protein (e.g., c-Maf or a small maf protein, such as p18) is modulated. In another embodiment, the activity of a protein that interacts with an NF-AT family protein (e.g., NIP45) is modulated. Combination methods, for example wherein the activities of a maf family protein and an NF-AT protein are modulated or the activities of a maf protein and an NF-AT-interacting protein are modulated, are also encompassed by the invention. Methods for modulating development of T helper type 1 (Th1) or T helper type 2 (Th2) subsets in a subject using agents that modulate transcription factor activity are also disclosed. NIP45 compositions, including isolated nucleic acid molecules encoding NIP45, antisense nucleic acid molecules, recombinant expression vectors containing a NIP45 nucleic acid molecule, host cells into which such expression vectors have been introduced and non-human transgenic animals carrying a NIP45 transgene, are also provided by the invention. The invention further provides isolated NIP45 proteins and peptides, NIP45 fusion proteins and anti-NIP45 antibodies. Methods of using the NIP45 compositions of the invention are also disclosed.

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METHODS AND COMPOSITIONS FOR REGULATING T CELL SUBSETS BY MODULATING TRANSCRIPTION FACTOR ACTIVITY

Background of the Invention

CD4+ T helper cells are not a homogeneous population but can be divided on the basis of cytokine secretion into at least two subsets termed T helper type 1 (Th1) and T helper type 2 (Th2) (see *e.g.*, Mosmann, T.R. *et al.* (1986) *J. Immunol.* 136:2348-2357; Paul, W.E. and Seder, R.A. (1994) *Cell* 76:241-251; Seder, R.A. and Paul, W.E. (1994) *Ann. Rev. Immunol.* 12:635-673). Th1 cells secrete interleukin-2 (IL-2) and interferon-γ (IFN-γ) while Th2 cells produce interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-10 (IL-10) and interleukin-13 (IL-13). Both subsets produce cytokines such as tumor necrosis factor (TNF) and granulocyte/macrophage-colony stimulating factor (GM-CSF). In addition to their different pattern of cytokine expression, Th1 and Th2 cells are thought to have differing functional activities. For example, Th1 cells are involved in inducing delayed type hypersensitivity responses, whereas Th2 cells are involved in providing efficient "help" to B lymphocytes and stimulating production of IgG1 and IgE antibodies.

There is now abundant evidence that the ratio of Th1 to Th2 cells is highly relevant to the outcome of a wide array of immunologically-mediated clinical diseases including autoimmune, allergic and infectious diseases. For example, in experimental leishmania infections in mice, animals that are resistant to infection mount predominantly a Th1 response, whereas animals that are susceptible to progressive infection mount predominantly a Th2 response (Heinzel, F.P., *et al.* (1989) *J. Exp. Med.* 169:59-72; Locksley, R. M. and Scott, P. (1992) *Immunoparasitology Today* 1:A58-A61). In murine schistosomiasis, a Th1 to Th2 switch is observed coincident with the release of eggs into the tissues by female parasites and is associated with a worsening of the disease condition (Pearce, E.J., *et al.* (1991) *J. Exp. Med.* 173:159-166; Grzych, J-M., *et al.* (1991) *J. Immunol.* 141:1322-1327; Kullberg, M.C., *et al.* (1992) *J. Immunol.* 148:3264-3270). Many human diseases, including chronic infections (such as with human immunodeficiency virus (HIV) and tuberculosis) and certain metastatic carcinomas, also are characterized by a Th1 to Th2 switch (see *e.g.*, Shearer, G.M. and Clerici, M. (1992) *Prog. Chem. Immunol.* 54:21-43; Clerici, M and Shearer, G.M. (1993) *Immunology Today* 14:107-111; Yamamura, M., *et al.* (1993) *J. Clin. Invest.* 91:1005-1010; Pisa, P., *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:7708-7712; Fauci, A.S. (1988) *Science* 239:617-623). Furthermore, certain autoimmune diseases have been shown to be associated with a predominant Th1 response. For example, patients

with rheumatoid arthritis have predominantly Th1 cells in synovial tissue (Simon, A.K., *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:8562-8566) and experimental autoimmune encephalomyelitis (EAE) can be induced by autoreactive Th1 cells (Kuchroo, V.K., *et al.* (1993) *J. Immunol.* 151:4371-4381).

The ability to alter or manipulate ratios of Th1 and Th2 subsets requires an understanding of the mechanisms by which the differentiation of CD4 T helper precursor cells (Thp), which secrete only IL-2, choose to become Th1 or Th2 effector cells. It is clear that the cytokines themselves are potent Th cell inducers and form an autoregulatory loop (see *e.g.*, Paul, W.E. and Seder, R.A. (1994) *Cell* 76:241-251; Seder, R.A. and Paul, W.E. (1994) *Ann. Rev. Immunol.* 12:635-673). Thus, IL-4 promotes the differentiation of Th2 cells while preventing the differentiation of precursors into Th1 cells, while IL-12 and IFN-γ have the opposite effect. One possible means therefore to alter Th1:Th2 ratios is to increase or decrease the level of selected cytokines. Direct administration of cytokines or antibodies to cytokines has been shown to have an effect on certain diseases mediated by either Th1 or Th2 cells. For example, administration of recombinant IL-4 or antibodies to IL-12 ameliorate EAE, a Th1-driven autoimmune disease (see Racke; M.K. *et al.* (1994) *J. Exp. Med.* 180:1961-1966; and Leonard, J.P. *et al.* (1995) *J. Exp. Med.* 181:381-386), while anti-IL-4 antibodies cure the Th2-mediated parasitic disease, *Leishmania major* (Sadick, M.D. *et al.* (1990) *J. Exp. Med.* 171:115-127). However, as therapeutic options, systemic administration of cytokines or antibodies may have unwanted side effects and, accordingly, alternative approaches to manipulating Th1/Th2 subsets are still needed.

The molecular basis for the tissue-specific expression of IL-4 in Th2 cells, or any T cell cytokine, has remained elusive. One possibility is the presence of repressor proteins that selectively silence cytokines. Transcriptional silencing has been well documented for bacteria, yeast and mammalian genes. Examples include *E. coli* thermoregulation genes (Goransson, M. *et al.* (1990) *Nature* 344:682-685), yeast α2 mating type genes (Keleher, C.A. *et al.* (1988) *Cell* 53:927-936) and mammalian MHC class I and TcRα genes (Weisman, J.D. and Singer, D.S. (1991) *Mol. Cell. Biol.* 11:4228-4234; Winoto, A. and Baltimore, D. (1989) *Cell* 59:649-655). Indeed, early experiments involving injection of IL-2 genomic DNA into *Xenopus* oocytes suggested the existence of a repressor protein for IL-2 in resting versus activated T cell extracts (Mouzaki, A. *et al.* (1991) *EMBO J.* 10:1399-1406). These studies suggested that the absence of IL-2 production in resting T cells was due to proteins that silenced the transcription of IL-2 by interacting with negative elements in the IL-2 promoter.

A second possibility is the existence of Th selective transactivators. Study of the transcriptional elements of cytokine genes has provided insight into the regulation of these cytokine genes. Analysis of the IL-4 cytokine promoter, for example, has revealed functionally critical sites for several transcription factors including members of the NF-AT and AP-1 families (Rooney, J.W. *et al.* (1995) *Immunity* 2:473-483; Szabo, S.J. *et al.* (1993) *Mol. Cell. Biol.* 13:4793-4805). NF-AT is a multisubunit transcription complex that contains a cyclosporin A sensitive cytoplasmic phosphoprotein and an inducible nuclear component composed of AP-1 family member proteins (Flanagan, W.M. *et al.* (1991) *Nature* 352:803-807; Jain, J. *et al.* (1992) *Nature* 356:801-804). Purification and cloning of NF-ATp revealed a region of limited sequence identity to the Rel Homology Domain (RHD) of the NFκB family of transcription factors (McCaffrey, P.G. *et al.* (1993) *Science* 262:750-754). Subsequent cloning and sequencing of three related genes, NF-ATc, NF-AT4/x/c3, and NF-AT3/c4 revealed similar domains. NF-AT family members share approximately 70% sequence similarity within this domain and approximately 18% sequence similarity to the RHD of the Rel/NFκB family of transcription factors. Consistent with their very limited sequence similarity in the RHD, there are marked differences in the behavior of NFκB and NF-AT proteins, and much less is known about the pathways that mediate transcriptional regulation of NF-AT target genes. However, considering that NF-AT family members can bind to and transactivate the promoters of multiple cytokine genes including IL-2 and IL-4 (Rooney, J. *et al.* (1995) *Immunity* 2:545-553; Szabo, S.J. *et al.* (1993) *Mol. Cell. Biol.* 13:4793-4805; Flanagan, W.M. *et al.* (1991) *Nature* 352:803-807; Northrop, J.P. *et al.* (1994) *Nature* 369:497), NF-AT proteins are not likely to be directly responsible for mediating Th1- or Th2-specific cytokine transcription.

Most, if not all, NF-AT binding sites in cytokine promoter regulatory regions are accompanied by nearby sites that bind auxiliary transcription factors, usually members of the AP-1 family. It has been shown that NF-AT and AP-1 proteins bind coordinately and cooperatively and are required for full activity of the IL-2 and IL-4 promoters. Different AP-1 proteins, specifically c-Jun, c-Fos, Fra-1, Fra-2, Jun B and Jun D, have been shown to bind to these sites (Rao, A. *et al.* (1994) *Immunol. Today* 15:274-281; Jain, J. *et al.* (1993) *Nature* 365:352-355; Boise, L.H. *et al.* (1993) *Mol. Cell. Biol.* 13:1911-1919; Rooney, J. *et al.* (1995) *Immunity* 2:545-553; Rooney, J. *et al.* (1995) *Mol. Cell. Biol.* 15:6299-6310). However, none of these AP-1 proteins is expressed in a Th1- or Th2-specific manner and there is no evidence for the differential recruitment of AP-1 family members to the IL-2 or IL-4 composite sites (Rooney, J. *et al.* (1995) *Mol. Cell. Biol.* 15:6299-6310). Thus, neither NF-AT proteins nor the AP-1 family members

c-Jun, c-Fos, Fra-1, Fra-2, Jun B and Jun D can account for the tissue-specific transcription of IL-4 in Th2 cells.

Summary of the Invention

This invention pertains to methods for regulating production of Th2-associated cytokines and for regulating Th1 or Th2 subsets by modulating the activity of one or more transcription factors that regulate expression of Th2-specific cytokine genes, and to compositions useful for such modulation As described further herein, it has now been discovered that the tissue-specific expression of IL-4 in Th2 cells is not due to a repressor protein but rather to a Th2-specific transactivator protein. The proto-oncogene c-Maf has now been demonstrated to be responsible for the tissue-specific expression of the Th2-associated cytokine interleukin-4. Moreover, ectopic expression of c-Maf in cells other than Th2 cells (*e.g.*, Th1 cells, B cells and non-lymphoid cells) leads to activation of the IL-4 promoter and, under appropriate conditions, production of endogenous IL-4. It further has been discovered that c-Maf and NF-AT synergize to activate Th2-associated cytokine gene expression.

Furthermore, a 45 kDa protein that interacts with members of the NF-AT family of proteins, termed NIP45 (for NF-AT Interacting Protein 45), has now been isolated and characterized. NIP45 was isolated based upon its ability to interact with the Rel Homology Domain (RHD) of NF-AT. NIP45 has been shown to synergize with NF-AT and c-Maf to stimulate cytokine gene expression. NIP45 potentiates gene expression mediated by c-Maf and NF-AT such that when all three factors (c-Maf, NF-AT and NIP45) are active in a cell, high levels of endogenous IL-4 production is stimulated. It still further has been discovered that a small maf protein lacking a transactivation domain, such as p18, can repress Th2-associated cytokine gene expression, *e.g.*, expression mediated by c-Maf.

Accordingly, this invention pertains to methods for modulating Th2-associated cytokines expression by modulating the expression or activity of one or more transcription factors that cooperate with an NF-AT family protein to regulate the expression of Th2-associated cytokine genes. In one embodiment, the transcription factor that cooperates with an NF-AT family protein to regulate the expression of a Th2-associated cytokine gene, and thus whose expression or activity is modulated, is a Th2-specific transcription factor (*e.g.*, a Th2-specific maf family protein). In another embodiment, the transcription factor that cooperates with an NF-AT family protein to regulate the expression of a Th2-associated cytokine gene, and thus whose expression or activity is modulated, is a maf family protein, such as c-Maf. In yet another

embodiment, the transcription factor that cooperates with an NF-AT family protein to regulate the expression of a Th2-associated cytokine gene, and thus whose expression or activity is modulated, is a protein that interacts with an NF-AT family protein, such as NIP45. In yet another embodiment, the expression or activity of a small maf protein, such as p18, is modulated. The methods of the invention may involve modulating the expression or activity of one transcription factor (*e.g.*, c-Maf or NIP45 or p18) or a combination of transcription factors (*e.g.*, c-Maf + NF-AT, or NF-AT + NIP45, or c-Maf + NF-AT + NIP45).

The modulatory methods of the invention generally involve contacting a cell with an agent that modulates the expression or activity of a transcription factor(s) such that production of the Th2-associated cytokine by a cell is modulated. In particular, preferred agents of the invention act intracellularly to modulate the activity of the transcription factor. In one embodiment, the modulatory method of the invention stimulates production of a Th2-associated cytokine. For example, Th2-associated cytokine production can be stimulated in Th1 cells, B cells or non-lymphoid cells. In another embodiment, the modulatory method of the invention inhibits production of a Th2-associated cytokine. A Th2-associated cytokine modulated in the method preferably is interleukin-4.

A variety of agents can be used to stimulate the expression or activity of a transcription factor that regulates expression of a Th2-associated cytokine gene. For example, a stimulatory agent of the invention can be a nucleic acid molecule encoding the transcription factor that is introduced into and expressed in the cell. Alternatively, chemical agents that enhance the expression or activity of the transcription facto can be used as stimulatory agents.

A variety of agents can be used to inhibit the expression or activity of a transcription factor that regulates expression of a Th2-associated cytokine gene. Examples of suitable inhibitory agents include antisense nucleic acid molecules that are complementary to a gene encoding the transcription factor, intracellular antibodies that bind the transcription factor (*e.g.*, in the cell nucleus), inhibitory forms of the transcription factor (*e.g.*, dominant negative forms) and chemical agents that inhibit the expression or activity of the transcription factor.

Combination methods, involving modulation of the expression or activity of two, three or more transcription factors that regulate Th2-associated cytokine gene expression, are also encompassed by the invention. Accordingly, in other embodiments of the invention, a cell is contacted with at least one additional agent that modulates the activity of at least one additional transcription factor that contributes to the regulation of

the Th2-associated cytokine gene. Preferably, the at least one additional transcription factor whose expression or activity is modulated is selected from the group consisting of NF-AT family proteins, NF-AT-interacting proteins, maf family proteins and AP-1 family proteins.

Cytokine production by a cell can be modulated *in vitro* or *in vivo* in accordance with the methods of the invention. In one embodiment, a cell is contacted with a modulating agent(s) *in vitro* and then the cell is administered to a subject to thereby regulate the development of Th1 and/or Th2 subsets in the subject. Accordingly, in another aspect, the invention provides methods for regulating the development of Th1 or Th2 subsets in a subject. In addition to the embodiment wherein *ex vivo* modified cells are administered to the subject, in another embodiment, these methods involve direct administration to the subject of an agent that modulates the activity of one or more transcription factors that regulate expression of a Th2-associated cytokine gene such that development of Th1 or Th2 cells in the subject is modulated.

The modulatory methods of the invention can be used to manipulate Th1:Th2 ratios in a variety of clinical situations. For example, inhibition of Th2 formation may be useful in allergic diseases, malignancies and infectious diseases whereas enhancement of Th2 formation may be useful in autoimmune diseases and organ transplantation.

Yet another aspect of the invention pertains to NIP45 compositions. This invention provides isolated compositions of NIP45 protein and isolated nucleic acid sequences encoding NIP45, other compositions related thereto and methods of use thereof. The amino acid sequence of NIP45 protein has been determined (shown in SEQ ID NO: 6) and a cDNA encoding NIP45 protein has been isolated (the nucleotide sequence of which is shown in SEQ ID NO: 5). In one aspect, the invention provides isolated nucleic acid molecules encoding NIP45, or fragments thereof. In one embodiment, the invention provides an isolated nucleic acid molecule comprising a nucleotide sequence encoding NIP45 protein. In another embodiment, the invention provides an isolated nucleic acid molecule comprising a nucleotide sequence encoding a protein, wherein the protein comprises an amino acid sequence that is homologous to the amino acid sequence of SEQ ID NO: 6 and interacts with the Rel Homology Domain of an NF-AT family protein. In yet another embodiment, the invention provides an isolated nucleic acid molecule which hybridizes under stringent conditions to a nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO: 5. In yet another embodiment, the invention provides an isolated nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO: 5. In still other embodiments, the invention

provides an isolated nucleic acid molecule encoding the amino acid sequence of SEQ ID NO: 6. Isolated nucleic acid molecules encoding NIP45 fusion proteins and isolated antisense nucleic acid molecules are also encompassed by the invention.

Another aspect of the invention pertains to vectors, such as recombinant expression vectors, containing a NIP45 nucleic acid molecule of the invention and host cells into which such vectors have been introduced. In one embodiment, such a host cell is used to produce NIP45 protein by culturing the host cell in a suitable medium. If desired, NIP45 protein can be then isolated from the host cell or the medium.

Still another aspect of the invention pertains to isolated NIP45 proteins, or portions thereof. In one embodiment, the invention provides an isolated NIP45 protein, or a portion thereof that interacts with an NF-AT family protein. In yet another embodiment, the invention provides an isolated protein which comprises an amino acid sequence homologous to the amino acid sequence of SEQ ID NO: 6 and that interacts with an NF-AT family protein. NIP45 fusion proteins are also encompassed by the invention.

The NIP45 proteins of the invention, or fragments thereof, can be used to prepare anti-NIP45 antibodies. Accordingly, the invention further provides an antibody that specifically binds NIP45 protein. In one embodiment, the antibody is monoclonal. In another embodiment, the antibody is labeled with a detectable substance.

The NIP45-encoding nucleic acid molecules of the invention can be used to prepare nonhuman transgenic animals that contain cells carrying a transgene encoding NIP45 protein or a portion of NIP45 protein. Accordingly, such transgenic animals are also provided by the invention. In one embodiment, the NIP45 transgene carried by the transgenic animal alters an endogenous gene encoding endogenous NIP45 protein (*e.g.*, a homologous recombinant animal).

Another aspect of the invention pertains to methods for detecting the presence of NIP45 protein or mRNA in a biological sample. To detect NIP45 protein or mRNA, the biological sample is contacted with an agent capable of detecting NIP45 protein (such as a labeled anti-NIP45 antibody) or NIP45 mRNA (such as a labeled nucleic acid probe capable of hybridizing to NIP45 mRNA) such that the presence of NIP45 protein or mRNA is detected in the biological sample.

Still another aspect of the invention pertains to methods for identifying compounds that modulate the activity or expression of NIP45 and methods for identifying compounds that modulate an interaction between NIP45 and an NF-AT family protein. Screening methods for identifying proteins that interact with NIP45 are also encompassed by the invention.

Yet another aspect of the invention pertains to compositions for expressing a maf protein in a host cell, such as an immune cells, and host cells expressing maf proteins. In one embodiment, these compositions include recombinant expression vectors that encode a maf family protein, wherein the maf-encoding sequences are operatively linked to regulatory sequences that direct expression of the maf family protein in a specific cell type, such as lymphoid cells (*e.g.*, T cells or B cells) or hematopoietic stem cells. In another embodiment, these compositions include host cells, such as host lymphoid cells (*e.g.*, host T cells or host B cells) or host hematopoietic stem cells, into which a recombinant expression vector encoding a maf family protein has been introduced.

The invention further provides transgenic animals that express a c-Maf protein. In a preferred embodiment, the transgenic animal is a mouse and the mouse overexpresses c-Maf in T cells, preferably using a trangene that comprises a CD4 promoter/enhancer operatively linked to a c-maf cDNA.

Brief Description of the Drawings

Figure 1A is a schematic of the cell fusion approach used to demonstrate that cytokine expression is not due to a repressor

Figure 1B is a reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of Il-2 and IL-4 cytokine, and control β-actin, mRNA expressed by an unfused Th1 clone (D1.1), an unfused Th2 clone (D10), Th1 and Th2 homokaryons and Th1-Th2 heterokaryons.

Figure 2A is a Northern blot analysis depicting expression of an isolated cDNA clone in Th1 cells, Th2 cells or B lymphoma cells. A control probe specific for GAPDH was used to show equal loading of RNA.

Figure 2B is a Northern blot analysis depicting upregulated expression of the isolated cDNA clone during *in vitro* differentiation of normal naive spleen cells into Th2 cells. Total RNA was isolated from cells harvested at the indicated time points. Culture supernatant at the appropriate dilution was measured for cytokine (IL-10) production by ELISA to determine differentiation into the Th1 or Th2 lineage.

Figure 3A is a bar graph depicting transactivation of the IL-4 promoter by c-Maf in a Th1 clone (AE7). AE7 cells were cotransfected with a wild-type IL-4 CAT reporter construct and either a control plasmid (pMEX-NeoI), a c-Maf expression plasmid (pMEX-Maf) or a c-Fos expression plasmid (pMEX-c-Fos). Half of each sample was stimulated 24 hours after transfection with antibodies to CD3. All samples were harvested 48 hours after transfection and relative CAT activities were determined.

Figure 3B is a photograph of a thin layer chromotography plate depicting the relative CAT activity in M12 B lymphoma cells cotransfected with a wild-type IL-4 CAT reporter construct and either two control plasmids (pMEX-NeoI and pREP4), a c-Maf expression plasmid and a control plasmid (pMEX-Maf and pREP4), a c-Fos expression plasmid and a control plasmid (pMEX-c-Fos and pREP4), a c-Jun expression plasmid and a control plasmid (pMEX-c-Jun and pREP4), a control plasmid and an NF-ATp expression plasmid (pMEX-NeoI and pREP-NF-ATp), a c-Maf expression plasmid and an NF-ATp expression plasmid (pMEX-Maf and pREP-NF-ATp) or a c-Fos expression plasmid and an NF-ATp expression plasmid (pMEX-c-Fos and pREP-NF-ATp). Half of each sample was stimulated 24 hours after transfection with PMA and ionomycin. All samples were harvested 48 hours after transfection and relative CAT activities were determined.

Figure 4 is a bar graph depicting endogenous production of IL-4 in M12 cells by ectopic expression of c-Maf and NF-ATp. Cells stably transfected with the indicated control or expression plasmids were either unstimulated or stimulated with PMA and ionomycin for 24 hours. 200 μl of supernatant from each sample was subjected to ELISA for cytokine quantitation.

Figure 5A is a photograph of a DNAse I footprint gel of the IL-4 promoter performed using nuclear extracts from Th2 (D10, CDC35) or Th1 (AE7, S53) clones harvested at the indicated time points after stimulation with anti-CD3 antibodies, which depicts a Th2-specific footprint immediately downstream of the putative MARE site in the IL-4 promoter. Two Th2-specific hypersensitive residues on the non-coding strand of the IL-4 promoter are indicated by *. Five lanes of a DNAse I digestion of the IL-4 promoter probe (without nuclear extract) and a Maxam-Gilbert A/G ladder were run next to the DNAse I treated samples.

Figure 5B is a schematic representation of the proximal regulatory region of the murine IL-4 promoter. The top portion shows the primary sequence of the murine IL-4 promoter. The numbers indicated are relative to the start site of transcription at +1. Asterisks denote the Th2-specific hypersensitive residues seen on DNAse I footprint. The bottom portion shows the sequence of the wild type (-59 to -28) oligonucleotide and the 4 bp mutants used in EMSA and the functional assays shown in Figures 6 and 7. Altered nucleotides are shown in lowercase bold and correspond to the numbering system shown in the top portion.

Figure 6 is a photograph of an electrophoretic mobility shift assay (EMSA) demonstrating that c-Maf but not c-Jun binds to the proximal IL-4 promoter and forms a

complex with NF-ATp. EMSA was performed using the indicated proteins and labeled double-stranded oligonucleotides.

Figure 7A is a bar graph (top) and a photograph of a thin layer chromotography plate (bottom) depicting the relative CAT activity in M12 cells co-transfected with a c-Maf expression vector and either the wild-type IL-4 CAT reporter construct or one of the 4 bp mutants shown in Figure 5B, demonstrating that transactivation of the IL-4 promoter by c-Maf maps to the MARE and Th2-specific footprint. The average of three independent experiments and one representative experiment are shown in the top and bottom portions, respectively.

Figure 7B is a photograph of an EMSA, performed using recombinant c-Maf, the IL-4 promoter (-59 to -27) probe and the indicated unlabeled double-stranded oligonucleotides as competitors, demonstrating that binding of recombinant c-Maf to the IL-4 promoter maps to the MARE and Th2-specific footprint.

Figure 8 is photograph of yeast colonies, in triplicate, transformed with the NIP45 plasmid and either NF-ATp-RHD as "bait" or control baits, Max, CDK2 or pEG202, together with the LacZ reporter plasmid pSH18, indicating that only those colonies containing the NIP45 plasmid and the NF-ATp-RHD bait expressed the LacZ reporter gene.

Figure 9 is a photograph of an immunoprecipitation/Western blot experiment demonstrating that NIP45 and NF-ATp interact in HepG2 cells.

Figure 10 is a schematic diagram comparing the structures of the original NIP45 cDNA clone isolated from the yeast two-hybrid screen (top) and the longest NIP45 cDNA clone isolated from a D10.G4 lambda zap II library (bottom).

Figure 11 depicts the nucleotide and predicted amino acid sequences of the original NIP45 cDNA isolate.

Figure 12 depicts the hydrophobicity plot of the NIP45 cDNA.

Figure 13 is a photograph of an RNA blot analysis of NIP45 transcript levels in various tissues.

Figure 14A is a photograph of immunofluorescence analysis of BHK cells transfected with an expression construct encoding an HA-epitope tagged NIP45 protein and probed with a monoclonal antibody specific for the HA peptide as the primary antibody and an indocarbocyanine labelled goat anti-mouse secondary reagent.

Figure 14B is a photograph of the same cells depicted in Figure 7A counterstained with the DNA staining dye Hoechst 33258.

Figure 14C is a photograph of immunofluorescence analysis of unstimulated BHK cells transfected with an expression construct encoding NF-AT4 and probed with

an anti-NF-AT4 specific antibody as the primary antibody and an indocarbocyanine labelled goat anti-mouse secondary reagent.

Figure 14D is a photograph of the same cells depicted in Figure 7C counterstained with the DNA staining dye Hoechst 33258.

Figure 14E is a photograph of immunofluorescence analysis of ionomycin-treated BHK cells transfected with an expression construct encoding NF-AT4 and probed with an anti-NF-AT4 specific antibody as the primary antibody and an indocarbocyanine labelled goat anti-mouse secondary reagent.

Figure 14F is a photograph of the same cells depicted in Figure 7D counterstained with the DNA staining dye Hoechst 33258.

Figure 15 is a photograph of CAT assay results (left) and a bar graph quantitating the relative fold induction of CAT activity (right) in HepG2 cells transfected with a 3X NF-AT-CAT reporter gene construct (containing three NF-AT binding sites) and either a control expression plasmid or an NF-AT family expression plasmid (NF-ATp, NF-ATc, NF-AT3 or NF-AT4), alone (-) or in combination with a NIP45 expression plasmid (+).

Figure 16 is a photograph of CAT assay results (left) and a bar graph quantitating the relative fold induction of CAT activity (right) in HepG2 cells transfected with an IL-4-CAT reporter gene construct (extending to -732 bp of the IL-4 promoter) and combinations of NF-ATp, NIP45 and/or c-Maf expression constructs, as indicated.

Figure 17 is a bar graph depicting the level of IL-4 (in pg/ml) in the supernatants of M12 B lymphoma cells transiently cotransfected with expression plasmids for NF-ATp, c-Maf and a pCI vector control (top bar) or expression plasmids for NF-ATp, c-Maf and NIP45 (bottom bar).

Figure 18 is a Northern blot analysis of transcripts expressed on day 0, 1, 3, 5 or 7 during *in vitro* differentiation of normal naive spleen cells into Th2 cells, depicting upregulated expression of c-maf over time and downregulated expression of p18 over time.

Figure 19 is a photograph of a thin layer chromotography plate depicting the relative CAT activity in M12 cells transfected with an IL-4 promoter reporter gene construct and either a c-Maf expression vector alone (5 μg), a p18 expression vector alone (10 μg) or a constant amount of c-Maf expression vector (5 μg) together with increasing amounts of a p18 expression vector alone (2.5, 5 or 10 μg), depicting repression of IL-4 promoter activity by p18.

Figure 20 is a schematic diagram of a c-maf transgene for overexpression in T cells using a CD4 promoter/enhancer to regulate expression of the c-maf cDNA.

Figure 21 is a bar graph depicting total cell numbers (x million) in lymph nodes (LN), spleen or thymus from either wild type mice or c-maf transfenic mice, demonstrating that c-maf transgenic mice exhibit decreased cell numbers in lymphoid organs.

Figure 22 is a bar graph depicting basal levels of serum IgE (ng/ml) in wild type mice or c-maf transfenic mice, demonstrating that c-maf transgenic mice exhibit increased basal levels of serum IgE.

Detailed Description of the Invention

In one aspect, this invention pertains to methods and compositions for regulating cytokine gene expression and T cell subsets by modulating transcription factor activity. The invention is based, at least in part, on the discovery that Th2-specific expression of the interleukin-4 gene does not result from the action of a specific repressor protein (as shown in Example 1) but rather from the action of a specific transactivator protein. As described further herein, the transcription factor responsible for Th2-specific expression of the interleukin-4 gene has now been identified as the c-Maf proto-oncoprotein, which is selectively expressed in differentiating and mature Th2 cells and absent from Th1 cells (see Example 2). Ectopic expression of c-Maf in cells that do not normally express it (such as Th1 cells and B cells) leads to transactivation of the IL-4 promoter (see Example 3) and, under appropriate conditions, to production of endogenous IL-4 (see Example 4). Moreover, a protein present in nuclear extracts of Th2 cells, but not Th1 cells, footprints the IL-4 promoter in the region of a maf response element (MARE) (see Example 5) and recombinant c-Maf binds to the IL-4 promoter *in vitro* (see Example 6). The ability of c-Maf to transactivate IL-4 maps to the MARE and Th2-specific footprint in the IL-4 promoter (see Example 7).

The invention further is based, at least in part, on the discovery of a protein that interacts with NF-AT and potentiates transcriptional activation by c-Maf and NF-AT. This protein, NIP45, was identified based upon its interaction with the Rel Homology Domain (RHD) of NF-AT (see Example 8). Coimmunoprecipitation experiments demonstrated that NIP45 and NF-AT interact *in vivo* in mammalian cells (see Example 9). The cDNA encoding NIP45 has been sequenced and characterized (see Example 10). Examination of the tissue expression pattern of NIP45 mRNA revealed that the NIP45 transcript is preferentially expressed in spleen, thymus and testis (see Example 11). Subcellular localization studies demonstrated that NIP45 protein is evenly distributed throughout the cell nucleus (see Example 12). Functional studies showed that NIP45 synergizes with NF-AT to stimulate transcription from promoters containing NF-AT

binding sites and, moreover, synergizes with NF-AT and c-Maf to stimulate transcription from the IL-4 promoter (see Example 13). Moreover, NIP45, NF-AT and c-Maf can act in concert to induce expression of the endogenous IL-4 gene in cells that do not normally express IL-4 (*e.g.*, B cells.) (see Example 14).

The invention still further is based, at least in part, on the discovery that a small maf protein, p18, that lacks an activation domain, can repress cytokine gene expression mediated by c-Maf. Differentiation of T helper cell precursors *in vitro* is associated with upregulation of *c-maf* gene expression and downregulation of p18 gene expression (see Example 15). Furthermore, coexpression of p18 with c-Maf represses IL-4 promoter activity, as compared to IL-4 promoter activity in the presence of c-Maf alone (see Example 16).

The invention is still further based on the generation of c-maf transgenic animals that overexpress c-Maf protein in T cells (see Example 17). These animals exhibit a phenotype very similar to transgenic animals that overexpress IL-4, namely small thymus and spleen, dramatic decreases in numbers of $CD^+/CD8^+$ (double-positive) thymocytes, decreased $CD4^+$ positive T cells and increased basal levels of serum IgE.

So that the invention may be more readily understood, certain terms are first defined.

As used herein, the term "Th2-associated cytokine" is intended to refer to a cytokine that is produced preferentially or exclusively by Th2 cells rather than by Th1 cells. Examples of Th2-associated cytokines include IL-4, IL-5, IL-6 and IL-13. A preferred Th2-associated cytokine whose production is modulated according to the methods of the invention is interleukin-4.

As used herein, the term "transcription factor" is intended to refer to a factor (e.g., a protein) that acts in the nucleus to regulate the transcriptional expression of a gene. The term "transcription factor" is intended to include factors that directly regulate transcription (e.g., have instrinsic transcriptional activation or inhibitory activity) and factors that indirectly regulate transcription (*e.g.*, through interaction with other factors that have intrinsic transcriptional activation or inhibitory activity).

As used herein, a transcription factor that "cooperates with a Nuclear Factor of Activated T cells family protein to regulate expression of a Th2-associated cytokine gene" is intended to refer to a transcription factor that synergizes or acts in concert with an NF-AT protein to regulate expression of a Th2-associated cytokine gene. That is, the expression of the Th2-associated cytokine gene (*e.g.*, IL-4) is greater in the presence of

both NF-AT and the cooperative transcription factor than in the presence of either alone. The cooperative transcription factor may or may not physically associate with NF-AT. Examples of transcription factors that cooperate with an NF-AT family protein to regulate expression of a Th2-associated cytokine gene include maf family proteins (*e.g.*, c-Maf) and NF-AT-interacting proteins (*e.g.*, NIP45).

As used herein, a transcription factor that "contributes to the regulation of a Th2-associated cytokine gene" is intended to refer to a transcription factor that participates in the transcriptional regulation of a Th2-associated cytokine gene, regardless of whether it cooperates with an NF-AT family protein. Transcription factors that cooperate with NF-AT to regulate the expression of a Th2-associated cytokine gene also are factors that contribute to the regulation of the Th2-associated cytokine gene. However, other transcription factors that do not cooperate with NF-AT also can contribute to the regulation of the Th2-associated cytokine gene. Examples of transcription factors that are thought to contribute to the regulation of Th2-associated cytokine genes include NF-AT family proteins, NF-AT-interacting proteins, maf family proteins, AP-1 family proteins, and Stat6 (Lederer, J. *et al.* (1996) *J. Exp. Med.* 184:397-406).

As used herein, the term "Th2-specific transcription factor" is intended to refer to a transcription factor that is expressed preferentially or exclusively in Th2 cells rather than in Th1 cells.

As used herein, the term "contacting" (*i.e.*, contacting a cell with an agent) is intended to include incubating the agent and the cell together *in vitro* (*e.g.*, adding the agent to cells in culture) and administering the agent to a subject such that the agent and cells of the subject are contacted *in vivo*.

As used herein, the various forms of the term "modulation" are intended to include stimulation (*e.g.*, increasing or upregulating a particular response or activity) and inhibition (*e.g.*, decreasing or downregulating a particular response or activity).

As used herein, the term "maf family protein" is intended to refer to a member of a sub-family of AP-1/CREB/ATF proteins that includes v-Maf, c-Maf, mafB, Nrl, mafK, mafF, mafG and p18. See *e.g.*, Nishizawa, M. *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86:7711-7715; Kataoka, K. *et al.* (1993) *J. Virol.* 67:2133-2141; Swaroop, A. *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:266-270; Fujiwara, K.T. *et al.* (1993) *Oncogene* 8:2371-2380; Igarashi, K. *et al.* (1995) *J. Biol. Chem.* 270:7615-7624; Andrews, N.C. *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:11488-11492; and Kataoka, K. *et al.* (1995) *Mol. Cell. Biol.* 15:2180-2190.

As used herein, the term "small maf protein" is intended to refer to a maf family protein that lacks a domain corresponding to the amino-terminal activation domain of c-Maf. Examples of small maf proteins include mafK, mafF, mafG and p18.

As used herein, the term "NF-AT family protein" (also referred to interchangeably as simple "NF-AT") is intended to refer to a member of the family of Nuclear Factors of Activated T cell transcription factors, including NF-ATp, NF-ATc, NF-AT4/x/c3 and NF-AT3/c4.

As used herein, the term "Rel Homology Domain of an NF-AT family protein" (abbreviated as RHD domain) is intended to refer to a domain within NF-AT family proteins having approximately 70% sequence similarity within the RHD of the Rel/NFκB family of transcription factors.

As used herein, the term "NF-AT-interacting protein" (used interchangeably with "a protein that interacts with an NF-AT family protein") is intended to refer to a factor that forms a physical association with an NF-AT family protein (e.g., co-immunoprecipitates with an NF-AT family protein). Preferably, the NF-AT-interacting protein interacts with the RHD of an NF-AT family protein. An example of an NF-AT-interacting protein is NIP45.

As used herein, the term "NIP45" is intended to include proteins having the amino acid sequence shown in SEQ ID NO: 6 (or encoded by the nucleotide sequence shown in SEQ ID NO: 5), as well as mammalian homologues thereof (e.g., human NIP45) and modified forms thereof (e.g., mutated or truncated forms) that retain the ability to interact with the RHD of NF-AT.

As used herein, the term "AP-1 family protein" is intended to refer to a protein that is a member of the AP-1 family of transcription factors, examples of which include c-Jun, c-Fos, Fra-1, Fra-2, Jun B and Jun D.

As used herein, the term "nucleic acid molecule" is intended to include DNA molecules (*e.g.*, cDNA or genomic DNA) and RNA molecules (*e.g.*, mRNA). The nucleic acid molecule may be single-stranded or double-stranded, but preferably is double-stranded DNA.

An used herein, an "isolated nucleic acid molecule" refers to a nucleic acid molecule that is free of gene sequences which naturally flank the nucleic acid in the genomic DNA of the organism from which the nucleic acid is derived (*i.e.*, gene sequences that are located adjacent to the isolated nucleic molecule in the genomic DNA of the organism from which the nucleic acid is derived). For example, in various embodiments, the isolated NIP45 nucleic acid molecule may contain less than about 5 kb, 4kb, 3kb, 2kb, 1 kb, 0.5 kb or 0.1 kb of nucleotide sequences which naturally flank

the nucleic acid molecule in genomic DNA of the cell from which the nucleic acid is derived. Moreover, an "isolated" nucleic acid molecule, such as a cDNA molecule, may be free of other cellular material.

As used herein, the term "hybridizes under stringent conditions" is intended to describe conditions for hybridization and washing under which nucleotide sequences at least 60 % homologous to each other typically remain hybridized to each other. Preferably, the conditions are such that at least sequences at least 65 %, more preferably at least 70 %, and even more preferably at least 75 % homologous to each other typically remain hybridized to each other. Such stringent conditions are known to those skilled in the art and can be found in *Current Protocols in Molecular Biology*, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. A preferred, non-limiting example of stringent hybridization conditions are hybridization in 6X sodium chloride/sodium citrate (SSC) at about 45°C, followed by one or more washes in 0.2 X SSC, 0.1% SDS at 50-65°C.

As used herein, a "naturally-occurring" nucleic acid molecule refers to an RNA or DNA molecule having a nucleotide sequence that occurs in nature (*e.g.*, encodes a natural protein).

As used herein, an "antisense" nucleic acid comprises a nucleotide sequence which is complementary to a "sense" nucleic acid encoding a protein, *e.g.*, complementary to the coding strand of a double-stranded cDNA molecule, complementary to an mRNA sequence or complementary to the coding strand of a gene. Accordingly, an antisense nucleic acid can hydrogen bond to a sense nucleic acid.

As used herein, the term "coding region" refers to regions of a nucleotide sequence comprising codons which are translated into amino acid residues, whereas the term "noncoding region" refers to regions of a nucleotide sequence that are not translated into amino acids (*e.g.*, 5' and 3' untranslated regions).

As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional DNA segments may be ligated. Another type of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (*e.g.*, bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (*e.g.*, non-episomal mammalian vectors) are integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to

herein as "recombinant expression vectors" or simply "expression vectors". In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" may be used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors, such as viral vectors (*e.g.*, replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions.

As used herein, the term "host cell" is intended to refer to a cell into which a nucleic acid of the invention, such as a recombinant expression vector of the invention, has been introduced. The terms "host cell" and "recombinant host cell" are used interchangeably herein. It should be understood that such terms refer not only to the particular subject cell but to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

As used herein, a nucleic acid molecule that is "in a form suitable for expression of the nucleic acid molecule in a cell" is intended to means that the nucleic acid molecule includes one or more regulatory sequences, selected on the basis of the host cells to be used for expression and the level of expression desired, which is operatively linked to the nucleic acid molecule to be expressed such that a protein encoded by the nucleic acid molecule is expressed in the host cell. Examples of such nucleic acid molecules include recombinant expression vectors containing nucleotide sequences encoding the protein to be expressed in the host cell.

As used herein, a "transgenic animal" refers to a non-human animal, preferably a mammal, more preferably a mouse, in which one or more of the cells of the animal includes a "transgene". The term "transgene" refers to exogenous DNA which is integrated into the genome of a cell from which a transgenic animal develops and which remains in the genome of the mature animal, for example directing the expression of an encoded gene product in one or more cell types or tissues of the transgenic animal.

As used herein, a "homologous recombinant animal" refers to a type of transgenic non-human animal, preferably a mammal, more preferably a mouse, in which an endogenous gene has been altered by homologous recombination between the endogenous gene and an exogenous DNA molecule introduced into a cell of the animal, *e.g.*, an embryonic cell of the animal, prior to development of the animal.

As used herein, an "isolated protein" refers to a protein that is substantially free of cellular material or culture medium when isolated from cells or produced by

recombinant DNA techniques, or chemical precursors or other chemicals when chemically synthesized.

As used herein, the term "antibody" is intended to include immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, *i.e.*, molecules that contain an antigen binding site which specifically binds (immunoreacts with) an antigen, such as Fab and $F(ab')_2$ fragments. The terms "monoclonal antibody" and "monoclonal antibody composition", as used herein, refer to a population of antibody molecules that contain only one species of an antigen binding site capable of immunoreacting with a particular epitope of an antigen. A monoclonal antibody composition thus typically displays a single binding affinity for a particular antigen with which it immunoreacts.

As used herein, an agent that "acts intracellularly to modulate the expression or activity of a transcription factor" is intended to refer to an agent that functions in an intracellular region of a cell, *e.g.*, the cytoplasm or nucleus, to modulate the expression or activity of the transcription factor. Thus, an agent that binds to the cell surface, such as an antibody, is not intended to be encompassed by the term "an agent that acts intracellularly to modulate the expression or activity of a transcription factor." Examples of agents that act intracellularly to modulate the expression or activity of a transcription factor include nucleic acid molecules that encode the transcription factor, antisense nucleic acid molecules, intracellular antibodies, dominant negative inhibitors and chemical agents that enter a cell and modulate (*i.e.*, stimulate or inhibit) transcription factor expression or activity.

As used herein, the term "intracellular binding molecule" is intended to include agents that act intracellularly to inhibit the expression or activity of a target protein of interest (*e.g.*, a transcription factor) by binding to the protein itself or to a nucleic acid (*e.g.*, an mRNA molecule) that encodes the protein. Examples of intracellular binding molecules include antisense nucleic acids, intracellular antibodies and dominant negative inhibitors.

Various aspects of the present invention are described in further detail in the following subsections.

I. Modulation of Th2-Associated Cytokine Production

The transcription factor responsible for the Th2-specific expression of the interleukin-4 gene has now been identified as the c-Maf proto-oncogene. Modulation of the expression and/or activity of c-Maf, therefore, provides a means to regulate the

production of interleukin-4. Since IL-4 itself serves an autoregulatory function in the development of Th2 cells (see *e.g.*, Paul, W.E. and Seder, R.A. (1994) *Cell* 76:241-251; Seder, R.A. and Paul, W.E. (1994) *Ann. Rev. Immunol.* 12:635-673), and thus production of IL-4 can lead to the production of additional Th2-associated cytokines such as IL-5, IL-6, IL-10 and IL-13 through further Th2 differentiation, modulation of c-Maf expression and/or activity provides a general approach for modulating production of Th2-associated cytokines.

The maf family of proteins are a sub-family of AP-1/CREB/ATF proteins that includes v-Maf, c-Maf, mafB, Nrl, mafK, mafF, mafG and p18. The *v-maf* oncogene was originally isolated from a spontaneous musculoaponeurotic fibrosarcoma of chicken and identified as the transforming gene of the avian retrovirus, AS42 (Nishizawa, M. *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86:7711-7715). V-*maf* encodes a 42 kd basic region/leucine zipper (b-zip) protein with homology to the *c-fos* and *c-jun* oncogenes. Its cellular homologue, the *c-maf* proto-oncogene has only two structural changes in the coding region from v-maf (Kataoka, K. *et al.* (1993) *J. Virol.* 67:2133-2141). The maf family includes c-Maf, mafB, a human retina-specific protein Nrl (Swaroop, A. *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:266-270), mafK, mafF, mafG and p18. The latter four, mafK, mafF, mafG and p18, each encode proteins that lack the amino terminal two thirds of c-Maf that contains the transactivating domain ("small maf proteins") (Fujiwara, K.T. *et al.* (1993) *Oncogene* 8:2371-2380; Igarashi, K. *et al.* (1995) *J. Biol. Chem.* 270:7615-7624; Andrews, N.C. *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:11488-11492; Kataoka, K. *et al.* (1995) *Mol. Cell. Biol.* 15:2180-2190). C-maf and other maf family members form homodimers and heterodimers with each other and with Fos and Jun, consistent with the known ability of the AP-1 proteins to pair with each other (Kerppola, T.K. and Curran, T. (1994) *Oncogene* 9:675-684; Kataoka, K. *et al.* (1994) *Mol. Cell. Biol.* 14:700-712). The DNA target sequence to which c-Maf homodimers bind, termed the c-Maf response element (MARE), is a 13 or 14 bp element which contains a core TRE (T-MARE) or CRE (C-MARE) palindrome respectively. Prior to the present invention, little was known about the function of maf family members, although c-Maf has been shown to stimulate transcription from the Purkinje neuron-specific promoter L7 (Kurscher, C. and Morgan, J.I. (1994) *Mol. Cell. Biol.* 15:246-254) and Nrl has been shown to drive expression of the QR1 retina-specific gene (Swaroop, A. *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:266-270). However, prior to the present invention, there have been no reports implicating c-Maf or other maf family members in the regulation of genes expressed in lymphoid cells or in cytokine gene expression in any tissue.

The small mafs have been shown to function as repressors of α and β-globin transcription when bound as homodimers but are essential as heterodimeric partners with the erythroid-specific factor p45NF-E2 to activate globin gene transcription (Kataoka, K. *et al.* (1995) *Mol. Cell. Biol.* 15:2180-2190; Igarashi, K. *et al.* (1994) *Nature* 367:568-572). MafK overexpression has been shown to induce erythroleukemia cell differentiation (Igarashi, K. *et al.* (1995) *Proc. Natl. Acad. Sci. USA* 92:7445-7449). The present invention provides evidence that small maf proteins *(e.g.*, p18) can modulate the expression of Th2-associated cytokine genes. Accordingly, modulation of the expression and/or activity of a small maf protein also provides a means to regulate the production of Th2-associated cytokine genes.

The present invention further provides an NF-AT-interacting protein, NIP45, that binds to and synergizes with NF-AT to regulate expression of a Th2-associated cytokine gene. NIP45 was identified based upon its interaction with the Rel Homology Domain of NF-ATp. NIP45 is described in further detail hereinbelow. Modulation of the expression and/or activity of an NF-AT-interacting protein, such as NIP45, thus also provides a means to regulate the production of Th2-associated cytokine genes.

Accordingly, this invention provides methods for modulating production of a Th2-associated cytokine by a cell by modulating the expression or activity of one or more transcription factors involved in Th2-associated cytokine gene expression. In one embodiment of the methods of the invention, a cell is contacted with an agent that modulates the expression or activity of a transcription factor such that such that production of the Th2-associated cytokine by a cell is modulated. In one embodiment, the transcription factor to be modulated is characterized as a transcription factor that cooperates with an NF-AT family protein to regulate expression of the Th2-associated cytokine gene (*e.g.*, c-Maf or NIP45). In another embodiment, the transcription factor to be modulated is a maf family protein (*e.g.*, c-Maf or a small maf protein, such as p18). In yet another embodiment, the transcription is an NF-AT-interacting protein (*e.g.*, NIP45). In preferred embodiments, the modulatory agents of the invention are characterized by acting intracellularly to modulate the activity of a transcription factor. In one embodiment, production of a Th2-associated cytokine by a cell is stimulated by contacting the cell with a stimulatory agent that stimulates transcription factor expression and/or activity. In another embodiment of the method of the invention, production of a Th2-associated cytokine by a cell is inhibited by contacting the cell with a inhibitory agent that inhibits transcription factor expression and/or activity.

As demonstrated in the Examples, although c-Maf is responsible for the tissue specificity of IL-4 gene expression, c-Maf acts synergistically with one or more

additional transcription factors to activate IL-4 gene transcription. In particular, c-Maf acts synergistically with an NF-AT protein to stimulate IL-4 gene expression. Moreover, NF-AT proteins and other members of the AP-1/CREB/ATF family of transcription factors have been demonstrated to be involved in regulating expression of both Th1- and Th2-associated cytokine genes. As further demonstrated in the Examples, a protein that interacts with NF-AT, NIP45, acts synergistically with NF-AT to stimulate expression from promoters containing NF-AT sites. Moreover, expression of a Th2-associated cytokine gene is potentiated by the presence of all three factors, c-Maf, NF-AT and NIP45. Accordingly, in another embodiment, the method of the invention for modulating Th2-associated cytokine production by a cell can comprises contacting the cell with multiple agents that modulate the expression or activity of transcription factors. Thus, in the methods of the invention in which a cell is contacted with a first agent, the methods can further comprise contacting the cell with one or more additional agents that modulate the activity of one or more additional transcription factors that contributes to the regulation of the Th2-associated cytokine gene. Preferably, the additonal agent(s) modulates the expression or activity or an additional transcription factor(s) selected from the group consisting of NF-AT family proteins, NF-AT-interacting proteins, maf family proteins and AP-1 family proteins.

As still further demonstrated in the Examples, a small maf protein (*e.g.*, p18) can repress Th2-associated cytokine gene expression mediated by positive transactivators (*e.g.*, c-Maf). Accordingly, in yet another embodiment, the method of the invention for modulating Th2-associated cytokine production by a cell comprises contacting the cell with an agent that modulates (*i.e.*, stimulates or inhibits) the expression or activity of a small maf protein, alone or in combination with agents that modulate the activity of other transcription factors, such as other maf family proteins, NF-AT family proteins or NF-AT-interacting proteins. Preferably, the small maf protein is p18. Other examples of small maf proteins include mafK, mafF and mafG.

A. *Stimulatory Agents*

According to the method of the invention, to stimulate Th2-associated cytokine production by a cell, the cell is contacted with a stimulatory agent that stimulates expression and/or activity of a transcription factor (*e.g.*, c-Maf, NIP45, p18) that regulates expression of a Th2-associated cytokine gene. Th2-associated cytokine production can be stimulated in cell types that do not normally express such cytokines, such as Th1 cells, B cells or non-lymphoid cells. Furthermore, Th2-associated cytokine

production can be stimulated in helper precursor cells (Thp) to promote their differentiation along the Th2 pathway instead of the Th1 pathway.

A preferred stimulatory agent is a nucleic acid molecule encoding a transcription factor that regulates expression of a Th2-associated cytokine gene, wherein the nucleic acid molecule is introduced into the cell in a form suitable for expression of the transcription factor in the cell. For example, a c-Maf cDNA is cloned into a recombinant expression vector and the vector is transfected into the cell. As demonstrated in Example 3, ectopic expression of a *c-maf* recombinant expression vector in Th1 cells, B cells or non-lymphoid cells leads to activation of the IL-4 promoter. Additionally, under appropriate conditions (discussed in further detail below), transcription of the endogenous IL-4 gene is stimulated, leading to IL-4 production by cells that do not normally express this cytokine (see Example 4).

To express a maf family protein in a cell, typically a maf family cDNA is first introduced into a recombinant expression vector using standard molecular biology techniques. A maf family cDNA can be obtained, for example, by amplification using the polymerase chain reaction (PCR) or by screening an appropriate cDNA library. The nucleotide sequences of maf family cDNAs are known in the art and can be used for the design of PCR primers that allow for amplification of a cDNA by standard PCR methods or for the design of a hybridization probe that can be used to screen a cDNA library using standard hybridization methods. Preferably, the maf family cDNA is that of the *c-maf* proto-oncogene. The nucleotide and predicted amino acid sequences of a mammalian (mouse) *c-maf* cDNA are disclosed in Kurscher C. and Morgan, J.I. (1995) *Mol. Cell. Biol.* 15:246-254 and deposited in the GenBank database at accession number S74567. This mammalian *c-maf* is highly homologous to the avian *v-maf* sequence (disclosed in Nishizawa, M.K. *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86:7711-7715 and GenBank accession numbers D28598 and D28596), indicating that *c-maf* is well conserved among species. *c-maf* cDNAs from other mammalian species, including humans, can be isolated using standard molecular biology techniques (*e.g.*, PCR or cDNA library screening) and primers or probes designed based upon the mouse or avian sequences. Human partial cDNA sequences homologous to the mouse c-maf cDNA are also deposited in the GenBank database at accession numbers H24189 and N75504. The sequences of other maf family members are also known in the art, for example MafB (Kataoka, K. *et al.* (1994) *Mol. Cell Biol.* 14:7581-91; GenBank accession number D28600), MafG (Kataoka *et al.* (1994) *Mol. Cell Biol.* 14:7581-91; GenBank accession numbers D28601 and D28602), MafF (GenBank accession number D16184)

and MafK (Igarashi, K. *et al.* (1995) *J. Biol. Chem.* 270:7615-7624; GenBank accession numbers D16187 and D42124).

Following isolation or amplification of a maf family cDNA, the DNA fragment is introduced into an expression vector. As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional DNA segments may be ligated. Another type of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (*e.g.*, bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (*e.g.*, non-episomal mammalian vectors) are integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "recombinant expression vectors" or simply "expression vectors". In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" may be used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors, such as viral vectors (*e.g.*, replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions

The recombinant expression vectors of the invention comprise a nucleic acid in a form suitable for expression of the nucleic acid in a host cell, which means that the recombinant expression vectors include one or more regulatory sequences, selected on the basis of the host cells to be used for expression and the level of expression desired, which is operatively linked to the nucleic acid sequence to be expressed. Within a recombinant expression vector, "operably linked" is intended to mean that the nucleotide sequence of interest is linked to the regulatory sequence(s) in a manner which allows for expression of the nucleotide sequence (*e.g.*, in an *in vitro* transcription/translation system or in a host cell when the vector is introduced into the host cell). The term "regulatory sequence" is intended to includes promoters, enhancers and other expression control elements (*e.g.*, polyadenylation signals). Such regulatory sequences are described, for example, in Goeddel; *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990). Regulatory sequences include those which direct constitutive expression of a nucleotide sequence in many types of host cell, those which direct expression of the nucleotide sequence only in

certain host cells (*e.g.*, tissue-specific regulatory sequences) or those which direct expression of the nucleotide sequence only under certain conditions (*e.g.*, inducible regulatory sequences).

It will be appreciated by those skilled in the art that the design of the expression vector may depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, *etc.* When used in mammalian cells, the expression vector's control functions are often provided by viral regulatory elements. For example, commonly used promoters are derived from polyoma virus, adenovirus, cytomegalovirus and Simian Virus 40. Non-limiting examples of mammalian expression vectors include pCDM8 (Seed, B., (1987) *Nature* 329:840) and pMT2PC (Kaufman *et al.* (1987), *EMBO J. 6*:187-195). A variety of mammalian expression vectors carrying different regulatory sequences are commercially available. For constitutive expression of the nucleic acid in a mammalian host cell, a preferred regulatory element is the cytomegalovirus promoter/enhancer. Moreover, inducible regulatory systems for use in mammalian cells are known in the art, for example systems in which gene expression is regulated by heavy metal ions (see *e.g.*, Mayo *et al.* (1982) *Cell* 29:99-108; Brinster *et al.* (1982) *Nature* 296:39-42; Searle *et al.* (1985) *Mol. Cell. Biol.* 5:1480-1489), heat shock (see *e.g.*, Nouer *et al.* (1991) in *Heat Shock Response*, e.d. Nouer, L. , CRC, Boca Raton , FL, pp167-220), hormones (see *e.g.*, Lee *et al.* (1981) *Nature* 294:228-232; Hynes *et al.* (1981) *Proc. Natl. Acad. Sci. USA* 78:2038-2042; Klock *et al.* (1987) *Nature* 329:734-736; Israel & Kaufman (1989) *Nucl. Acids Res.* 17:2589-2604; and PCT Publication No. WO 93/23431), FK506-related molecules (see *e.g.*, PCT Publication No. WO 94/18317) or tetracyclines (Gossen, M. and Bujard, H. (1992) *Proc. Natl. Acad. Sci. USA* 89:5547-5551; Gossen, M. *et al.* (1995) *Science* 268:1766-1769; PCT Publication No. WO 94/29442; and PCT Publication No. WO 96/01313). Still further, many tissue-specific regulatory sequences are known in the art, including the albumin promoter (liver-specific; Pinkert *et al.* (1987) *Genes Dev.* 1:268-277), lymphoid-specific promoters (Calame and Eaton (1988) *Adv. Immunol.* 43:235-275), in particular promoters of T cell receptors (Winoto and Baltimore (1989) *EMBO J.* 8:729-733) and immunoglobulins (Banerji *et al.* (1983) *Cell* 33:729-740; Queen and Baltimore (1983) *Cell* 33:741-748), neuron-specific promoters (*e.g.*, the neurofilament promoter; Byrne and Ruddle (1989) *Proc. Natl. Acad. Sci. USA* 86:5473-5477), pancreas-specific promoters (Edlund *et al.* (1985) *Science* 230:912-916) and mammary gland-specific promoters (*e.g.*, milk whey promoter; U.S. Patent No. 4,873,316 and European Application Publication No. 264,166). Developmentally-regulated promoters are also encompassed, for example the murine hox promoters (Kessel and Gruss (1990)

Science 249:374-379) and the α-fetoprotein promoter (Campes and Tilghman (1989) *Genes Dev.* 3:537-546).

Vector DNA can be introduced into mammalian cells via conventional transfection techniques. As used herein, the various forms of the term "transfection" are intended to refer to a variety of art-recognized techniques for introducing foreign nucleic acid (*e.g.*, DNA) into mammalian host cells, including calcium phosphate co-precipitation, DEAE-dextran-mediated transfection, lipofection, or electroporation. Suitable methods for transfecting host cells can be found in Sambrook *et al.* (*Molecular Cloning: A Laboratory Manual*, 2nd Edition, Cold Spring Harbor Laboratory press (1989)), and other laboratory manuals.

For stable transfection of mammalian cells, it is known that, depending upon the expression vector and transfection technique used, only a small fraction of cells may integrate the foreign DNA into their genome. In order to identify and select these integrants, a gene that encodes a selectable marker (*e.g.*, resistance to antibiotics) is generally introduced into the host cells along with the gene of interest. Preferred selectable markers include those which confer resistance to drugs, such as G418, hygromycin and methotrexate. Nucleic acid encoding a selectable marker may be introduced into a host cell on a separate vector from that encoding a maf family protein or, more preferably, on the same vector. Cells stably transfected with the introduced nucleic acid can be identified by drug selection (*e.g.*, cells that have incorporated the selectable marker gene will survive, while the other cells die).

Nucleic acid molecules encoding other transcription factors that regulate Th2-associated cytokine gene expression, in form suitable for expression of the transcription factor in a host cell, can be prepared as described above using nucleotide sequences known in the art or disclosed herein. The nucleotide sequences can be used for the design of PCR primers that allow for amplification of a cDNA by standard PCR methods or for the design of a hybridization probe that can be used to screen a cDNA library using standard hybridization methods. The nucleotide and predicted amino acid sequence of NIP45 are disclosed in SEQ ID NOs: 5 and 6, respectively. The nucleotide and predicted amino acid sequences of small maf proteins, including p18, mafK, mafF and mafG, are known in the art (see *e.g.*, Fujiwara, K.T. *et al.* (1993) *Oncogene* 8:2371-2380; Igarashi, K. *et al.* (1995) *J. Biol. Chem.* 270:7615-7624; Andrews, N.C. *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:11488-11492; Kataoka, K. *et al.* (1995) *Mol. Cell. Biol.* 15:2180-2190). The nucleotide and predicted amino acid sequences of NF-AT family proteins, including NF-ATp, NF-ATc, NF-AT4/x/c3 and NF-AT3/c4, are known in the art. Four NF-AT family members have been identified (see *e.g.*, Emmel, E.A. *et*

al. (1989) *Science* 246:1617-1620; Flanagan, W.M. *et al.* (1991) *Nature* 352:803-807; Jain, J. *et al.* (1993) *Nature* 365:352-355; McCaffrey, P.G. *et al.* (1993) *Science* 262:750-754; Rao, A. (1994) *Immunol. Today* 15:274-281; Northrop, J.P. *et al.* (1994) *Nature* 369:497). Preferably, the NF-AT cDNA is that of NF-ATp. The nucleotide and predicted amino acid sequences of a mammalian NF-ATp cDNA are disclosed in McCaffrey, P.G. *et al.* (1993) *Science* 262:750-754. The nucleotide and predicted amino acid sequences of a mammalian NF-ATc cDNA are disclosed in Northrop, J.P. *et al.* (1994) *Nature* 369:497 and deposited in the GenBank database at accession number U08015. The nucleotide and predicted amino acid sequences of mammalian NF-AT3 and NF-AT4 cDNAs are disclosed in Hoey, T. *et al.* (1995) *Immunity* 2:461-472. The nucleotide and predicted amino acid sequences of AP-1 family proteins are known in the art. For example, the nucleotide and predicted amino acid sequences of human *c-fos* are disclosed in van Straaten, F. *et al.* (1983) *Proc. Natl. Acad. Sci. USA* 80:3183-3187. The nucleotide and predicted amino acid sequences of human *c-jun* are disclosed in Bohmann, D. *et al.* (1987) *Science* 238:1386-1392. The nucleotide and predicted amino acid sequences of human *jun-B* and *jun-D* are disclosed in Nomura, N. *et al.* (1990) *Nucl. Acids Res.* 18:3047-3048. The nucleotide and predicted amino acid sequences of human *fra-1* and *fra-2* are disclosed in Matsui, M. *et al.* (1990) *Oncogene* 5:249-255.

Another form of a stimulatory agent for stimulating expression of a Th2-associated cytokine in a cell is a chemical compound that stimulates the expression or activity of an endogenous transcription factor that regulates expression of Th2-associated cytokine genes in the cell (*e.g.*, a maf family protein, such as c-Maf or p18, or a protein that interacts with NF-AT, such as NIP45). Such compounds can be identified using screening assays that select for compounds that stimulate the expression or activity of the transcription factor. Examples of suitable screening assays are described in further detail in subsection V below.

In addition to use of a first agent that stimulates the expression or activity of a first transcription factor that regulates Th2-associated cytokine gene expression, the stimulatory methods of the invention can involve the use of one or more additional agents that stimulate the expression or activity of one or more additional transcription factors that contribute to regulating the expression of a Th1- or Th2-associated cytokine gene. In Example 4, it is shown that stimulation of the expression of endogenous IL-4 in M12 B lymphoma cells required the introduction into the cells of both a c-Maf expression vector and an NF-AT expression vector, thereby demonstrating that c-Maf and NF-AT act synergistically to activate IL-4 transcription, with c-maf responsible for the tissue-specificity of expression. In Example 14, it further is shown that stimulation

of the expression of endogenous IL-4 in M12 B lymphoma cells is potentiated by coexpression of c-Maf, NF-AT and NIP45. While the skilled artisan will appreciate that certain cells may express sufficient amounts of endogenous c-Maf, NF-AT and/or NIP45 such that use of a single agent alone may be sufficient to stimulate expression of a Th2-associated cytokine gene, in certain situations and with certain cell types it may be necessary to stimulate multiple transcription factors, such as both c-Maf and NF-AT, both c-Maf and NIP45, or all three proteins (c-Maf, NF-AT and NIP45), to achieve the desired stimulation of Th2-associated cytokine production.

Accordingly, in the stimulatory method of the invention in which a cell is contacted with a first agent that stimulates the expression or activity of a first transcription factor, the method can further comprise contacting the cell with at least one additional agent that stimulates the expression or activity of at least additional transcription factors that contribute to regulating the expression of a Th1- or Th2-associated cytokine gene. Preferably, the at least one additional transcription factor whose expression or activity is modulated is selected from the group consisting of NF-AT family proteins, NF-AT-interacting proteins, maf family proteins and AP-1 family proteins. For example, a stimulatory method of the invention can involve the use of a first agent that stimulates the expression or activity of c-Maf and a second agent that stimulates the expression or activity of either an NF-AT family protein or a protein that interacts with an NF-AT family protein (*e.g.*, NIP45). In another embodiment, the stimulatory methods of the invention involve the use of a first agent that stimulates the expression or activity of c-Maf, a second agent that stimulates the expression or activity of an NF-AT family protein and a third agent that stimulates the expression or activity of a protein that interacts with an NF-AT family protein (*e.g.*, NIP45). A preferred agent for stimulating NF-AT or NIP45 activity in a cell is a recombinant expression encoding NF-AT or NIP45, respectively, wherein the recombinant expression vector is introduced into the cell and NF-AT or NIP45 is expressed in the cell. NF-AT- and NIP45-encoding expression vectors can be prepared and introduced into cells as described above for c-Maf expression vectors.

Alternative to use of an NF-AT or NIP45 cDNA to stimulate the activity of NF-AT or NIP45 in a cell, one or more chemical compounds that stimulate NF-AT or NIP45 activity in a cell can be used as a second (or additional) agent in a stimulatory method of the invention. Compounds that stimulate NF-AT activity in cells are known in the art (for a review see Rao, A. (1994) *Immunol. Today* 15:274-281). For example, stimulation of certain cells with the phorbol ester phorbol myristate acetate (PMA) and a calcium ionophore (*e.g.*, ionomycin) results in translocation of NF-ATs to the cell

nucleus (see *e.g.*, Flanagan, W.M. *et al.* (1991) *Nature* 352:803-807; Jain, J. *et al.* (1993) *Nature* 365:352-355). Additionally, stimulation of T cells through the T cell receptor (TcR), for example with an anti-CD3 antibody, results in activation of NF-AT in the T cells.

In addition to NF-AT proteins, AP-1 family members, including c-Jun, c-Fos, Fra-1, Fra-2, Jun B and Jun D, have been shown to be involved in regulating the expression of both Th1- and Th2-associated cytokine genes (*e.g.*, IL-2 and IL-4) (see *e.g.*, Rao, A. *et al.* (1994) *Immunol. Today* 15:274-281; Jain, J. *et al.* (1993) *Nature* 365:352-355; Boise, L.H. *et al.* (1993) *Mol. Cell. Biol.* 13:1911-1919; Rooney, J. *et al.* (1995) *Immunity* 2:545-553; Rooney, J. *et al.* (1995) *Mol. Cell. Biol.* 15:6299-6310). Although these factors are not responsible for the Th1/Th2 specificity of expression of the cytokine genes, and these factors do not appear to synergize with c-Maf in regulating IL-4 gene expression (see the Examples), AP-1 family members have been shown to increase IL-4 expression in Th2 cells (see *e.g.*, Rooney, J. *et al.* (1995) *Immunity* 2:545-553). Accordingly, in certain circumstances it may be beneficial, in addition to stimulating c-Maf activity (and possibly NF-AT activity), also to stimulate the activity of an AP-1 family protein. Accordingly, in one embodiment, the stimulatory methods of the invention involve the use of a first agent that stimulates the expression or activity of c-Maf and a second agent that stimulates the expression or activity of an AP-1 protein. In another embodiment, the invention involves the use of a first agent that stimulates the expression or activity of c-Maf, a second agent that stimulates the expression or activity of an NF-AT protein and a third agent that stimulates the expression or activity of an AP-1 protein. NIP45 activity also can be modulated in combination with maf, AP-1 and/or NF-AT family proteins.

A preferred agent for stimulating AP-1 activity in a cell is a recombinant expression encoding an AP-1 protein, wherein the recombinant expression vector is introduced into the cell and the AP-1 protein is expressed in the cell. AP-1-encoding expression vectors can be prepared and introduced into cells as described above for c-Maf expression vectors. Alternatively, one or more chemical compounds that stimulate AP-1 activity in a cell can be used as additional agents in a stimulatory method of the invention. Compounds that stimulate AP-1 activity in cells are known in the art, including PMA/calcium ionophore (*e.g.*, ionomycin) and anti-CD3 antibodies.

B. *Inhibitory Agents*

According to the method of the invention, to inhibit Th2-associated cytokine production by a cell, the cell is contacted with an inhibitory agent that inhibits expression and/or activity of a transcription factor (*e.g.*, c-Maf, NIP45, p18) that regulates expression of a Th2-associated cytokine gene. In one embodiment, Th2-associated cytokine production by a cell is inhibited by contacting the cell with an agent that modulates the expression or activity of a transcription factor that cooperates with an NF-AT family protein to regulate expression of a Th2-associated cytokine gene. In another embodioment, Th2-associated cytokine production by a cell is inhibited by contacting the cell with an agent that modulates the expression or activity of a Th2-specific transcription factor, preferably c-Maf. In another embodiment, Th2-associated cytokine production by a cell is inhibited by contacting the cell with an agent that modulates the expression or activity of a protein that interacts with an NF-AT family protein, preferably NIP45. In yet another embodiment, Th2-associated cytokine production by a cell is inhibited by contacting the cell with an agent that modulates the expression or activity of a small maf protein. As discussed above for stimulatory methods, the inhibitory methods of the invention can comprise contacting the cell with two or more agents that modulate the expression or activity of two or more transcription factors that regulate Th2-associated cytokine gene expression, including maf family proteins, NF-AT family proteins, NF-AT-interacting proteins and AP-1 family proteins.

Th2-associated cytokine production can be inhibited in, for example, Th2 cells or in helper precursor cells (Thp) to promote their differentiation along the Th1 pathway instead of the Th2 pathway. Inhibitory agents of the invention can be, for example, intracellular binding molecules that act to inhibit the expression or activity of the transcription factor. As used herein, the term "intracellular binding molecule" is intended to include molecules that act intracellularly to inhibit the expression or activity of a protein by binding to the protein or to a nucleic acid (*e.g.*, an mRNA molecule) that encodes the protein. Examples of intracellular binding molecules, described in further detail below, include antisense nucleic acids, intracellular antibodies and dominant negative inhibitors.

In one embodiment, an inhibitory agent of the invention is an antisense nucleic acid molecule that is complementary to a gene encoding a transcription factor (*e.g.*, a maf family protein, such as c-Maf or p18, or an NF-AT-interacting protein, such as NIP45), or to a portion of said gene, or a recombinant expression vector encoding said antisense nucleic acid molecule. The use of antisense nucleic acids to downregulate the expression of a particular protein in a cell is well known in the art (see *e.g.*, Weintraub, H. *et al.*, Antisense RNA as a molecular tool for genetic analysis, *Reviews - Trends in*

Genetics, Vol. 1(1) 1986; Askari, F.K. and McDonnell, W.M. (1996) *N. Eng. J. Med.* 334:316-318; Bennett, M.R. and Schwartz, S.M. (1995) *Circulation* 92:1981-1993; Mercola, D. and Cohen, J.S. (1995) *Cancer Gene Ther.* 2:47-59; Rossi, J.J. (1995) *Br. Med. Bull.* 51:217-225; Wagner, R.W. (1994) *Nature* 372:333-335). An antisense nucleic acid molecule comprises a nucleotide sequence that is complementary to the coding strand of another nucleic acid molecule (*e.g.*, an mRNA sequence) and accordingly is capable of hydrogen bonding to the coding strand of the other nucleic acid molecule. Antisense sequences complementary to a sequence of an mRNA can be complementary to a sequence found in the coding region of the mRNA, the 5' or 3' untranslated region of the mRNA or a region bridging the coding region and an untranslated region (*e.g.*, at the junction of the 5' untranslated region and the coding region). Furthermore, an antisense nucleic acid can be complementary in sequence to a regulatory region of the gene encoding the mRNA, for instance a transcription initiation sequence or regulatory element. Preferably, an antisense nucleic acid is designed so as to be complementary to a region preceding or spanning the initiation codon on the coding strand or in the 3' untranslated region of an mRNA. An antisense nucleic acid for inhibiting in a cell the expression of a transcription factor discussed herein can be designed based upon the nucleotide sequence of the transcription factor, as disclosed herein or known in the art, constructed according to the rules of Watson and Crick base pairing.

An antisense nucleic acid can exist in a variety of different forms. For example, the antisense nucleic acid can be an oligonucleotide that is complementary to only a portion of a maf family gene. An antisense oligonucleotides can be constructed using chemical synthesis procedures known in the art. An antisense oligonucleotide can be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed between the antisense and sense nucleic acids, *e.g.* phosphorothioate derivatives and acridine substituted nucleotides can be used. To inhibit transcription factor expression in cells in culture, one or more antisense oligonucleotides can be added to cells in culture media, typically at 200 μg oligonucleotide/ml.

Alternatively, an antisense nucleic acid can be produced biologically using an expression vector into which a nucleic acid has been subcloned in an antisense orientation (*i.e.*, nucleic acid transcribed from the inserted nucleic acid will be of an antisense orientation to a target nucleic acid of interest). Regulatory sequences operatively linked to a nucleic acid cloned in the antisense orientation can be chosen

which direct the expression of the antisense RNA molecule in a cell of interest, for instance promoters and/or enhancers or other regulatory sequences can be chosen which direct constitutive, tissue specific or inducible expression of antisense RNA. The antisense expression vector is prepared as described above for recombinant expression vectors, except that the cDNA (or portion thereof) is cloned into the vector in the antisense orientation. The antisense expression vector can be in the form of, for example, a recombinant plasmid, phagemid or attenuated virus. The antisense expression vector is introduced into cells using a standard transfection technique, as described above for recombinant expression vectors.

In another embodiment, an antisense nucleic acid for use as an inhibitory agent is a ribozyme. Ribozymes are catalytic RNA molecules with ribonuclease activity which are capable of cleaving a single-stranded nucleic acid, such as an mRNA, to which they have a complementary region (for reviews on ribozymes see *e.g.*, Ohkawa, J. *et al.* (1995) *J. Biochem.* 118:251-258; Sigurdsson, S.T. and Eckstein, F. (1995) *Trends Biotechnol.* 13:286-289; Rossi, J.J. (1995) *Trends Biotechnol.* 13:301-306; Kiehntopf, M. *et al.* (1995) *J. Mol. Med.* 73:65-71). A ribozyme having specificity for mRNA encoding a transcription factor discussed herein can be designed based upon the nucleotide sequence of the transcription factor. For example, a derivative of a *Tetrahymena* L-19 IVS RNA can be constructed in which the base sequence of the active site is complementary to the base sequence to be cleaved in a *c-maf* mRNA or other transcription factor mRNA. See for example U.S. Patent Nos. 4,987,071 and 5,116,742, both by Cech *et al.* Alternatively, *c-maf* mRNA (or other transcription factor mRNA) can be used to select a catalytic RNA having a specific ribonuclease activity from a pool of RNA molecules. See for example Bartel, D. and Szostak, J.W. (1993) *Science* 261: 1411-1418.

Another type of inhibitory agent that can be used to inhibit the expression and/or activity of a Maf protein in a cell is an intracellular antibody specific for a transcription factor discussed herein. The use of intracellular antibodies to inhibit protein function in a cell is known in the art (see *e.g.*, Carlson, J. R. (1988) *Mol. Cell. Biol.* 8:2638-2646; Biocca, S. *et al.* (1990) *EMBO J.* 9:101-108; Werge, T.M. *et al.* (1990) *FEBS Letters* 274:193-198; Carlson, J.R. (1993) *Proc. Natl. Acad. Sci. USA* 90:7427-7428; Marasco, W.A. *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:7889-7893; Biocca, S. *et al.* (1994) *Bio/Technology* 12:396-399; Chen, S-Y. *et al.* (1994) *Human Gene Therapy* 5:595-601; Duan, L *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:5075-5079; Chen, S-Y. *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:5932-5936; Beerli, R.R. *et al.* (1994) *J. Biol. Chem.* 269:23931-23936; Beerli, R.R. *et al.* (1994) *Biochem. Biophys. Res. Commun.* 204:666-672; Mhashilkar, A.M. *et al.* (1995) *EMBO J.* 14:1542-1551; Richardson, J.H. *et al.* (1995)

Proc. Natl. Acad. Sci. USA 92:3137-3141; PCT Publication No. WO 94/02610 by Marasco *et al.*; and PCT Publication No. WO 95/03832 by Duan *et al.*).

To inhibit protein activity using an intracellular antibody, a recombinant expression vector is prepared which encodes the antibody chains in a form such that, upon introduction of the vector into a cell, the antibody chains are expressed as a functional antibody in an intracellular compartment of the cell. For inhibition of transcription factor activity according to the inhibitory methods of the invention, preferably an intracellular antibody that specifically binds the transcription factor is expressed within the nucleus of the cell. Nuclear expression of an intracellular antibody can be accomplished by removing from the antibody light and heavy chain genes those nucleotide sequences that encode the N-terminal hydrophobic leader sequences and adding nucleotide sequences encoding a nuclear localization signal at either the N- or C-terminus of the light and heavy chain genes (see *e.g.*, Biocca, S. *et al.* (1990) *EMBO J.* 9:101-108; Mhashilkar, A. M. *et al.* (1995) *EMBO J.* 14:1542-1551). A preferred nuclear localization signal to be used for nuclear targeting of the intracellular antibody chains is the nuclear localization signal of SV40 Large T antigen (see Biocca, S. *et al.* (1990) *EMBO J.* 9:101-108; Mhashilkar, A. M. *et al.* (1995) *EMBO J.* 14:1542-1551).

To prepare an intracellular antibody expression vector, antibody light and heavy chain cDNAs encoding antibody chains specific for the target protein of interest, *e.g.*, a Maf family protein or other transcription factor discussed herein, are isolated, typically from a hybridoma that secretes a monoclonal antibody specific for the maf protein. Preparation of antisera against Maf family proteins has been described in the art (see *e.g.*, Fujiwara, K.T. *et al.* (1993) *Oncogene* 8:2371-2380; Kataoka, K. *et al.* (1993) *J. Virol.* 67:2133-2141; Kerppola, T.K. and Curran, T. (1994) *Oncogene* 9:675-684; Igarashi, K *et al.* (1995) *Proc. Natl. Acad. Sci. USA* 92:7445-7449). Anti-Maf protein antibodies can be prepared by immunizing a suitable subject, (*e.g.*, rabbit, goat, mouse or other mammal) with a Maf protein immunogen. An appropriate immunogenic preparation can contain, for examples, recombinantly expressed Maf protein or a chemically synthesized Maf peptide. The preparation can further include an adjuvant, such as Freund's complete or incomplete adjuvant, or similar immunostimulatory agent. Antibody-producing cells can be obtained from the subject and used to prepare monoclonal antibodies by standard techniques, such as the hybridoma technique originally described by Kohler and Milstein (1975, *Nature* 256:495-497) (see also, Brown *et al.* (1981) *J. Immunol* 127:539-46; Brown *et al.* (1980) *J Biol Chem* 255:4980-83; Yeh *et al.* (1976) *PNAS* 76:2927-31; and Yeh *et al.* (1982) *Int. J. Cancer* 29:269-75). The technology for producing monoclonal antibody hybridomas is well

known (see generally R. H. Kenneth, in *Monoclonal Antibodies: A New Dimension In Biological Analyses*, Plenum Publishing Corp., New York, New York (1980); E. A. Lerner (1981) *Yale J. Biol. Med.*, 54:387-402; M. L. Gefter *et al.* (1977) *Somatic Cell Genet.*, 3:231-36). Briefly, an immortal cell line (typically a myeloma) is fused to lymphocytes (typically splenocytes) from a mammal immunized with a maf protein immunogen as described above, and the culture supernatants of the resulting hybridoma cells are screened to identify a hybridoma producing a monoclonal antibody that binds specifically to the Maf protein. Any of the many well known protocols used for fusing lymphocytes and immortalized cell lines can be applied for the purpose of generating an anti-Maf protein monoclonal antibody (see, *e.g.*, G. Galfre *et al.* (1977) *Nature* 266:550-52; Gefter *et al. Somatic Cell Genet.*, cited *supra*; Lerner, *Yale J. Biol. Med.*, cited *supra*; Kenneth, *Monoclonal Antibodies*, cited *supra*). Moreover, the ordinary skilled worker will appreciate that there are many variations of such methods which also would be useful. Typically, the immortal cell line (*e.g.*, a myeloma cell line) is derived from the same mammalian species as the lymphocytes. For example, murine hybridomas can be made by fusing lymphocytes from a mouse immunized with an immunogenic preparation of the present invention with an immortalized mouse cell line. Preferred immortal cell lines are mouse myeloma cell lines that are sensitive to culture medium containing hypoxanthine, aminopterin and thymidine ("HAT medium"). Any of a number of myeloma cell lines may be used as a fusion partner according to standard techniques, *e.g.*, the P3-NS1/1-Ag4-1, P3-x63-Ag8.653 or Sp2/O-Ag14 myeloma lines. These myeloma lines are available from the American Type Culture Collection (ATCC), Rockville, Md. Typically, HAT-sensitive mouse myeloma cells are fused to mouse splenocytes using polyethylene glycol ("PEG"). Hybridoma cells resulting from the fusion are then selected using HAT medium, which kills unfused and unproductively fused myeloma cells (unfused splenocytes die after several days because they are not transformed). Hybridoma cells producing a monoclonal antibody that specifically binds the maf protein are identified by screening the hybridoma culture supernatants for such antibodies, *e.g.*, using a standard ELISA assay.

Alternative to preparing monoclonal antibody-secreting hybridomas, a monoclonal antibody that binds to a transcription factor discussed herein can be identified and isolated by screening a recombinant combinatorial immunoglobulin library (*e.g.*, an antibody phage display library) with the protein, or a peptide thereof, to thereby isolate immunoglobulin library members that bind specifically to the protein. Kits for generating and screening phage display libraries are commercially available (*e.g.*, the Pharmacia *Recombinant Phage Antibody System*, Catalog No. 27-9400-01; and

the Stratagene *SurfZAP*™ *Phage Display Kit*, Catalog No. 240612). Additionally, examples of methods and reagents particularly amenable for use in generating and screening antibody display library can be found in, for example, Ladner *et al.* U.S. Patent No. 5,223,409; Kang *et al.* International Publication No. WO 92/18619; Dower *et al.* International Publication No. WO 91/17271; Winter *et al.* International Publication WO 92/20791; Markland *et al.* International Publication No. WO 92/15679; Breitling *et al.* International Publication WO 93/01288; McCafferty *et al.* International Publication No. WO 92/01047; Garrard *et al.* International Publication No. WO 92/09690; Fuchs *et al.* (1991) *Bio/Technology* 9:1370-1372; Hay *et al.* (1992) *Hum Antibod Hybridomas* 3:81-85; Huse *et al.* (1989) *Science* 246:1275-1281; Griffiths *et al.* (1993) *EMBO J* 12:725-734; Hawkins *et al.* (1992) *J Mol Biol* 226:889-896; Clarkson *et al.* (1991) *Nature* 352:624-628; Gram *et al.* (1992) *PNAS* 89:3576-3580; Garrad *et al.* (1991) *Bio/Technology* 9:1373-1377; Hoogenboom *et al.* (1991) *Nuc Acid Res* 19:4133-4137; Barbas *et al.* (1991) *PNAS* 88:7978-7982; and McCafferty *et al. Nature* (1990) 348:552-554.

Once a monoclonal antibody specific for the transcription factor of interest has been identified (*e.g.*, either a hybridoma-derived monoclonal antibody or a recombinant antibody from a combinatorial library), DNAs encoding the light and heavy chains of the monoclonal antibody are isolated by standard molecular biology techniques. For hybridoma derived antibodies, light and heavy chain cDNAs can be obtained, for example, by PCR amplification or cDNA library screening. For recombinant antibodies, such as from a phage display library, cDNA encoding the light and heavy chains can be recovered from the display package (*e.g.*, phage) isolated during the library screening process. Nucleotide sequences of antibody light and heavy chain genes from which PCR primers or cDNA library probes can be prepared are known in the art. For example, many such sequences are disclosed in Kabat, E.A., *et al.* (1991) *Sequences of Proteins of Immunological Interest, Fifth Edition*, U.S. Department of Health and Human Services, NIH Publication No. 91-3242 and in the "Vbase" human germline sequence database.

Once obtained, the antibody light and heavy chain sequences are cloned into a recombinant expression vector using standard methods. As discussed above, the sequences encoding the hydrophobic leaders of the light and heavy chains are removed and sequences encoding a nuclear localization signal (*e.g.*, from SV40 Large T antigen) are linked in-frame to sequences encoding either the amino- or carboxy terminus of both the light and heavy chains. The expression vector can encode an intracellular antibody in one of several different forms. For example, in one embodiment, the vector encodes

full-length antibody light and heavy chains such that a full-length antibody is expressed intracellularly. In another embodiment, the vector encodes a full-length light chain but only the VH/CH1 region of the heavy chain such that a Fab fragment is expressed intracellularly. In the most preferred embodiment, the vector encodes a single chain antibody (scFv) wherein the variable regions of the light and heavy chains are linked by a flexible peptide linker (*e.g.*, $(Gly_4Ser)_3$) and expressed as a single chain molecule. To inhibit transcription factor activity in a cell, the expression vector encoding the transcription factor-specific intracellular antibody is introduced into the cell by standard transfection methods, as discussed hereinbefore.

Yet another form of an inhibitory agent of the invention is an inhibitory form of a transcription factor discussed herein (*e.g.*, a maf protein), also referred to herein as a dominant negative inhibitor. The maf family of proteins are known to homodimerize and to heterodimerize with other AP-1 family members, such as Fos and Jun (see *e.g.*, Kerppola, T.K. and Curran, T. (1994) *Oncogene* 9:675-684; Kataoka, K. *et al.* (1994) *Mol. Cell. Biol.* 14:700-712). One means to inhibit the activity of transcription factors that form dimers is through the use of a dominant negative inhibitor that has the ability to dimerize with functional transcription factors but that lacks the ability to activate transcription (see *e.g.*, Petrak, D. *et al.* (1994) *J. Immunol.* 153:2046-2051). By dimerizing with functional transcription factors, such dominant negative inhibitors can inhibit their functional activity. This process may occur naturally as a means to regulate gene expression. For example, there are a number of "small" maf proteins, such as *mafK*, *mafF*, *mafG* and *p18*, which lack the amino terminal two thirds of c-Maf that contains the transactivating domain (Fujiwara, K.T. *et al.* (1993) *Oncogene* 8:2371-2380; Igarashi, K. *et al.* (1995) *J. Biol. Chem.* 270:7615-7624; Andrews, N.C. *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:11488-11492; Kataoka, K. *et al.* (1995) *Mol. Cell. Biol.* 15:2180-2190). Homodimers of the small maf proteins act as negative regulators of transcription (Igarashi, K. *et al.* (1994) *Nature* 367:568-572) and three of the small maf proteins (MafK, MafF and MafG) have been shown to competitively inhibit transactivation mediated by the v-Maf oncoprotein (Kataoka, K. *et al.* (1996) *Oncogene* 12:53-62). Additionally, MafB has been identified as an interaction partner of Ets-1 and shown to inhibit Ets-1-mediated transactivation of the transferrin receptor and to inhibit erythroid differentiation (Sieweke, M.H. *et al.* (1996) *Cell* 85:49-60).

Accordingly, an inhibitory agent of the invention can be a form of a Maf protein that has the ability to dimerize with c-Maf but that lacks the ability to activate transcription. This dominant negative form of a Maf protein may be, for example, a small Maf protein (*e.g.*, MafK, MafF, MafG) that naturally lacks a transactivation

domain, MafB or a mutated form of c-Maf in which the transactivation domain has been removed. Such dominant negative Maf proteins can be expressed in cells using a recombinant expression vector encoding the Maf protein, which is introduced into the cell by standard transfection methods. To express a mutant form of c-Maf lacking a transactivation domain, nucleotide sequences encoding the amino terminal transactivation domain of c-Maf are removed from the *c-maf* cDNA by standard recombinant DNA techniques. Preferably, at least amino acids 1-122 are removed. More preferably, at least amino acids 1-187, or amino acids 1-257, are removed. Nucleotide sequences encoding the basic-leucine zipper region are maintained. The truncated cDNA is inserted into a recombinant expression vector, which is then introduced into a cell to allow for expression of the truncated c-maf, lacking a transactivation domain, in the cell.

Yet another type of inhibitory agent that can be used to inhibit the expression and/or activity of a maf protein in a cell is chemical compound that inhibits the expression or activity of an endogenous maf family protein in the cell. Such compounds can be identified using screening assays that select for compounds that inhibit the expression or activity of a maf family protein. Examples of suitable screening assays are described in further detail in subsection V below.

As discussed above with regard to stimulatory agents, the inhibitory methods of the invention can involve the use of one or more additional inhibitory agents that inhibit the expression or activity of one or more additional transcription factors that contributes to regulating the expression of a Th1- or Th2-associated cytokine gene. For example, in one embodiment, the inhibitory method of the invention comprises contacting a cell with a first agent that inhibits the expression or activity a maf family protein and a second agent that inhibits the expression or activity of an NF-AT family protein or an NF-AT-interacting protein (*e.g.*, NIP45). In another embodiment, the inhibitory method of the invention comprises contacting a cell with a first agent that inhibits the expression or activity a maf family protein and a second agent that inhibits the expression or activity of an AP-1 family protein. In yet another embodiment, the inhibitory method of the invention comprises contacting a cell with a first agent that inhibits the expression or activity a maf family protein, a second agent that inhibits the expression or activity of an NF-AT family protein and a third agent that inhibits the expression or activity of an NF-AT-interacting protein (*e.g.*, NIP45). Examples of types of inhibitory agents for inhibiting NF-AT, NF-AT-interacting and AP-1 proteins include antisense nucleic acids, intracellular antibodies, dominant negative inhibitors and chemical compounds that inhibit the endogenous proteins, as described above. Regarding the latter, it is known in

the art that the nuclear translocation of NF-ATp is inhibited by the immunosuppressive drugs cyclosporin A and FK506 (see *e.g.*, Rao, A. (1994) *Immunol. Today* 15:274-281; Rao, A. (1995) *J. Leukoc. Biol.* 57:536-542). Accordingly, in one embodiment of the inhibitory method, an immunosuppressive drug such as cyclosporin A or FK506 (or other related drug that inhibits the calcineurin pathway) is used in combination with an agent that inhibits the expression or activity of c-Maf.

The method of the invention for modulating production of Th2-associated cytokines by a cell can be practiced either *in vitro* or *in vivo* (the latter is discussed further in the following subsection). For practicing the method *in vitro*, cells can be obtained from a subject by standard methods and incubated (*i.e.*, cultured) *in vitro* with a stimulatory or inhibitory agent of the invention to stimulate or inhibit, respectively, the production of Th2-associated cytokines. For example, peripheral blood mononuclear cells (PBMCs) can be obtained from a subject and isolated by density gradient centrifugation, *e.g.*, with Ficoll/Hypaque. Specific cell populations can be depleted or enriched using standard methods. For example, monocytes/macrophages can be isolated by adherence on plastic. T cells or B cells can be enriched or depleted, for example, by positive and/or negative selection using antibodies to T cell or B cell surface markers, for example by incubating cells with a specific primary monoclonal antibody (mAb), followed by isolation of cells that bind the mAb using magnetic beads coated with a secondary antibody that binds the primary mAb. Peripheral blood or bone marrow derived hematopoietic stem cells can be isolated by similar techniques using stem cell-specific mAbs (*e.g.*, anti-CD34 mAbs). Specific cell populations can also be isolated by fluorescence activated cell sorting according to standard methods. Monoclonal antibodies to cell-specific surface markers known in the art and many are commercially available.

When a stimulatory agent is used *in vitro*, resulting in stimulation of the production of Th2-associated cytokines, in particular IL-4, the cytokine(s) can be recovered from the culture supernatant for further use. For example, the culture supernatant, or a purified fraction thereof, can be applied to T cells in culture to influence the development of Th1 or Th2 cells *in vitro*. Alternatively, the culture supernatant, or a purified fraction thereof, can be administered to a subject to influence the development of Th1 vs. Th2 responses in the subject.

Moreover, cells treated *in vitro* with either a stimulatory or inhibitory agent can be administered to a subject to influence the development of a Th1 vs. Th2 response in the subject. Accordingly, in another embodiment, the method of the invention for

modulating the production of Th2-associated cytokines by a cell further comprises administering the cell to a subject to thereby modulate development of Th1 or Th2 cells in a subject. Preferred cell types for *ex vivo* modification and readministration include T cells, B cells and hematopoietic stem cells. For administration to a subject, it may be preferable to first remove residual agents in the culture from the cells before administering them to the subject. This can be done for example by a Ficoll/Hypaque gradient centrifugation of the cells. For further discussion of *ex vivo* genetic modification of cells followed by readministration to a subject, see also U.S. Patent No. 5,399,346 by W.F. Anderson *et al.*

II. Methods for Modulating Development of Th1 or Th2 Cells in a Subject

Another aspect of the invention pertains to a method for modulating development of Th1 or Th2 cells in a subject. The term "subject" is intended to include living organisms in which an immune response can be elicited. Preferred subjects are mammals. Examples of subjects include humans, monkeys, dogs, cats, mice, rats, cows, horses, goats and sheep. As discussed above, one way to modulate Th1/Th2 ratios in a subject is to treat cells (*e.g.*, T cells, B cells or hematopoietic stem cells) *ex vivo* with one or more modulatory agents of the invention, such that production of a Th2-associated cytokine by the cells is modulated, followed by administration of the cells to the subject. In another embodiment, Th1/Th2 ratios are modulated in a subject by administering to the subject an agent that modulates the activity of a transcription factor that regulates expression of a Th2-associated cytokine gene such that development of Th1 or Th2 cells in the subject is modulated. In a preferred embodiment, the transcription factor is a maf family protein, preferably a c-Maf protein or a small maf protein (*e.g.*, p18). In another preferred embodiment, the transcription factor is a protein that interacts with an NF-AT family protein, preferably NIP45. Preferably, the Th2-associated cytokine is IL-4. Development of a Th2 response in the subject can be promoted by administration of one or more stimulatory agents of the invention, whereas development of a Th1 response in the subject can be promoted by administration of one or more inhibitory agents of the invention. As discussed above, in certain situations it may be desirable, in addition to modulating the activity of multiple transcription factors (*e.g.*, combinations of a maf family protein, an NF-AT family protein, an NF-AT-interacting protein and/or an AP-1 family protein).

For stimulatory or inhibitory agents that comprise nucleic acids (including recombinant expression vectors encoding transcription factors, antisense RNA, intracellular antibodies or dominant negative inhibitors), the agents can be introduced

into cells of the subject using methods known in the art for introducing nucleic acid (*e.g.*, DNA) into cells *in vivo*. Examples of such methods include:

Direct Injection: Naked DNA can be introduced into cells *in vivo* by directly injecting the DNA into the cells (see *e.g.*, Acsadi *et al.* (1991) *Nature* 332:815-818; Wolff *et al.* (1990) *Science* 247:1465-1468). For example, a delivery apparatus (*e.g.*, a "gene gun") for injecting DNA into cells *in vivo* can be used. Such an apparatus is commercially available (*e.g.*, from BioRad).

Receptor-Mediated DNA Uptake: Naked DNA can also be introduced into cells *in vivo* by complexing the DNA to a cation, such as polylysine, which is coupled to a ligand for a cell-surface receptor (see for example Wu, G. and Wu, C.H. (1988) *J. Biol. Chem.* 263:14621; Wilson *et al.* (1992) *J. Biol. Chem.* 267:963-967; and U.S. Patent No. 5,166,320). Binding of the DNA-ligand complex to the receptor facilitates uptake of the DNA by receptor-mediated endocytosis. A DNA-ligand complex linked to adenovirus capsids which naturally disrupt endosomes, thereby releasing material into the cytoplasm can be used to avoid degradation of the complex by intracellular lysosomes (see for example Curiel *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:8850; Cristiano *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:2122-2126).

Retroviruses: Defective retroviruses are well characterized for use in gene transfer for gene therapy purposes (for a review see Miller, A.D. (1990) *Blood* 76:271). A recombinant retrovirus can be constructed having a nucleotide sequences of interest incorporated into the retroviral genome. Additionally, portions of the retroviral genome can be removed to render the retrovirus replication defective. The replication defective retrovirus is then packaged into virions which can be used to infect a target cell through the use of a helper virus by standard techniques. Protocols for producing recombinant retroviruses and for infecting cells *in vitro* or *in vivo* with such viruses can be found in Current Protocols in Molecular Biology, Ausubel, F.M. *et al.* (eds.) Greene Publishing Associates, (1989), Sections 9.10-9.14 and other standard laboratory manuals. Examples of suitable retroviruses include pLJ, pZIP, pWE and pEM which are well known to those skilled in the art. Examples of suitable packaging virus lines include ψ Crip, ψCre, ψ2 and ψAm. Retroviruses have been used to introduce a variety of genes into many different cell types, including epithelial cells, endothelial cells, lymphocytes, myoblasts, hepatocytes, bone marrow cells, *in vitro* and/or *in vivo* (see for example Eglitis, *et al.* (1985) *Science* 230:1395-1398; Danos and Mulligan (1988) *Proc. Natl. Acad. Sci. USA* 85:6460-6464; Wilson *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:3014-3018; Armentano *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87:6141-6145; Huber *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:8039-8043; Ferry *et al.* (1991) *Proc. Natl. Acad.*

Sci. USA 88:8377-8381; Chowdhury *et al.* (1991) *Science* 254:1802-1805; van Beusechem *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:7640-7644; Kay *et al.* (1992) *Human Gene Therapy* 3:641-647; Dai *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:10892-10895; Hwu *et al.* (1993) *J. Immunol.* 150:4104-4115; U.S. Patent No. 4,868,116; U.S. Patent No. 4,980,286; PCT Application WO 89/07136; PCT Application WO 89/02468; PCT Application WO 89/05345; and PCT Application WO 92/07573). Retroviral vectors require target cell division in order for the retroviral genome (and foreign nucleic acid inserted into it) to be integrated into the host genome to stably introduce nucleic acid into the cell. Thus, it may be necessary to stimulate replication of the target cell.

Adenoviruses: The genome of an adenovirus can be manipulated such that it encodes and expresses a gene product of interest but is inactivated in terms of its ability to replicate in a normal lytic viral life cycle. See for example Berkner *et al.* (1988) *BioTechniques* 6:616; Rosenfeld *et al.* (1991) *Science* 252:431-434; and Rosenfeld *et al.* (1992) *Cell* 68:143-155. Suitable adenoviral vectors derived from the adenovirus strain Ad type 5 dl324 or other strains of adenovirus (*e.g.*, Ad2, Ad3, Ad7 *etc.*) are well known to those skilled in the art. Recombinant adenoviruses are advantageous in that they do not require dividing cells to be effective gene delivery vehicles and can be used to infect a wide variety of cell types, including airway epithelium (Rosenfeld *et al.* (1992) cited *supra*), endothelial cells (Lemarchand *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:6482-6486), hepatocytes (Herz and Gerard (1993) *Proc. Natl. Acad. Sci. USA* 90:2812-2816) and muscle cells (Quantin *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:2581-2584). Additionally, introduced adenoviral DNA (and foreign DNA contained therein) is not integrated into the genome of a host cell but remains episomal, thereby avoiding potential problems that can occur as a result of insertional mutagenesis in situations where introduced DNA becomes integrated into the host genome (*e.g.*, retroviral DNA). Moreover, the carrying capacity of the adenoviral genome for foreign DNA is large (up to 8 kilobases) relative to other gene delivery vectors (Berkner *et al.* cited *supra*; Haj-Ahmand and Graham (1986) *J. Virol.* 57:267). Most replication-defective adenoviral vectors currently in use are deleted for all or parts of the viral E1 and E3 genes but retain as much as 80 % of the adenoviral genetic material.

Adeno-Associated Viruses: Adeno-associated virus (AAV) is a naturally occurring defective virus that requires another virus, such as an adenovirus or a herpes virus, as a helper virus for efficient replication and a productive life cycle. (For a review see Muzyczka *et al.* *Curr. Topics in Micro. and Immunol.* (1992) 158:97-129). It is also one of the few viruses that may integrate its DNA into non-dividing cells, and exhibits a

high frequency of stable integration (see for example Flotte *et al.* (1992) *Am. J. Respir. Cell. Mol. Biol.* 7:349-356; Samulski *et al.* (1989) *J. Virol.* 63:3822-3828; and McLaughlin *et al.* (1989) *J. Virol.* 62:1963-1973). Vectors containing as little as 300 base pairs of AAV can be packaged and can integrate. Space for exogenous DNA is limited to about 4.5 kb. An AAV vector such as that described in Tratschin *et al.* (1985) *Mol. Cell. Biol.* 5:3251-3260 can be used to introduce DNA into cells. A variety of nucleic acids have been introduced into different cell types using AAV vectors (see for example Hermonat *et al.* (1984) *Proc. Natl. Acad. Sci. USA* 81:6466-6470; Tratschin *et al.* (1985) *Mol. Cell. Biol.* 4:2072-2081; Wondisford *et al.* (1988) *Mol. Endocrinol.* 2:32-39; Tratschin *et al.* (1984) *J. Virol.* 51:611-619; and Flotte *et al.* (1993) *J. Biol. Chem.* 268:3781-3790).

The efficacy of a particular expression vector system and method of introducing nucleic acid into a cell can be assessed by standard approaches routinely used in the art. For example, DNA introduced into a cell can be detected by a filter hybridization technique (*e.g.*, Southern blotting) and RNA produced by transcription of introduced DNA can be detected, for example, by Northern blotting, RNase protection or reverse transcriptase-polymerase chain reaction (RT-PCR). The gene product can be detected by an appropriate assay, for example by immunological detection of a produced protein, such as with a specific antibody, or by a functional assay to detect a functional activity of the gene product, such as an enzymatic assay.

A modulatory agent, such as a chemical compound that stimulates or inhibits endogenous transcription factor activity, can be administered to a subject as a pharmaceutical composition. Such compositions typically comprise the modulatory agent and a pharmaceutically acceptable carrier. As used herein the term "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. For example, solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents;

antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL^{TM} (BASF, Parsippany, NJ) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyetheylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating the active compound in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Oral compositions generally include an inert diluent or an edible carrier. They can be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches, or capsules. Oral compositions can also be prepared using a fluid carrier for use as a mouthwash, wherein the compound in the fluid carrier is applied orally and swished and expectorated or swallowed. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

In one embodiment, the active compounds are prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These may be prepared according to methods known to those skilled in the art, for example, as described in U.S. Patent No. 4,522,811.

It is especially advantageous to formulate oral or parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of individuals.

III. Applications of the Methods of the Invention

Identification of transcription factors that control the production of IL-4, and hence continued formation of Th2 cells, allows for selective manipulation of T cell subsets in a variety of clinical situations using the modulatory methods of the invention. The stimulatory methods of the invention (*i.e.*, methods that use a stimulatory agent) result in production of Th2-associated cytokines, with concomitant promotion of a Th2 response and downregulation of a Th1 response. In contrast, the inhibitory methods of the invention (*i.e.*, methods that use an inhibitory agent) inhibit the production of Th2-associated cytokines, with concomitant downregulation of a Th2 response and promotion of a Th1 response. Thus, to treat a disease condition wherein a Th2 response is beneficial, a stimulatory method of the invention is selected such that Th2 responses are promoted while downregulating Th1 responses. Alternatively, to treat a disease condition wherein a Th1 response is beneficial, an inhibitory method of the invention is selected such that Th2 responses are downregulated while promoting Th1 responses. Application of the methods of the invention to the treatment of disease conditions may result in cure of the condition, a decrease in the type or number of symptoms associated with the condition, either in the long term or short term (*i.e.*, amelioration of the condition) or simply a transient beneficial effect to the subject.

Numerous disease conditions associated with a predominant Th1 or Th2-type response have been identified and could benefit from modulation of the type of response mounted in the individual suffering from the disease condition. Application of the immunomodulatory methods of the invention to such diseases is described in further detail below.

A. *Allergies*

Allergies are mediated through IgE antibodies whose production is regulated by the activity of Th2 cells and the cytokines produced thereby. In allergic reactions, IL-4 is produced by Th2 cells, which further stimulates production of IgE antibodies and activation of cells that mediate allergic reactions, *i.e.*, mast cells and basophils. IL-4 also plays an important role in eosinophil mediated inflammatory reactions. Accordingly, the inhibitory methods of the invention can be used to inhibit the production of Th2-associated cytokines, and in particular IL-4, in allergic patients as a means to downregulate production of pathogenic IgE antibodies. An inhibitory agent may be directly administered to the subject or cells (*e.g.*, Thp cells or Th2 cells) may be obtained from the subject, contacted with an inhibitory agent *ex vivo*, and readministered

to the subject. Moreover, in certain situations it may be beneficial to coadminister to the subject the allergen together with the inhibitory agent or cells treated with the inhibitory agent to inhibit (*e.g.*, desensitize) the allergen-specific response. The treatment may be further enhanced by administering other Th1-promoting agents, such as the cytokine IL-12 or antibodies to Th2-associated cytokines (*e.g.*, anti-IL-4 antibodies), to the allergic subject in amounts sufficient to further stimulate a Th1-type response.

B. *Cancer*

The expression of Th2-promoting cytokines has been reported to be elevated in cancer patients (see *e.g.*, Yamamura, M., *et al.* (1993) *J. Clin. Invest.* 91:1005-1010; Pisa, P., *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:7708-7712) and malignant disease is often associated with a shift from Th1 type responses to Th2 type responses along with a worsening of the course of the disease. Accordingly, the inhibitory methods of the invention can be used to inhibit the production of Th2-associated cytokines in cancer patients, as a means to counteract the Th1 to Th2 shift and thereby promote an ongoing Th1 response in the patients to ameliorate the course of the disease. The inhibitory method can involve either direct administration of an inhibitory agent to a subject with cancer or *ex vivo* treatment of cells obtained from the subject (*e.g.*, Thp or Th2 cells) with an inhibitory agent followed by readministration of the cells to the subject. The treatment may be further enhanced by administering other Th1-promoting agents, such as the cytokine IL-12 or antibodies to Th2-associated cytokines (*e.g.*, anti-IL-4 antibodies), to the recipient in amounts sufficient to further stimulate a Th1-type response.

C. *Infectious Diseases*

The expression of Th2-promoting cytokines also has been reported to increase during a variety of infectious diseases, including HIV infection, tuberculosis, leishmaniasis, schistosomiasis, filarial nematode infection and intestinal nematode infection (see *e.g.*; Shearer, G.M. and Clerici, M. (1992) *Prog. Chem. Immunol.* 54:21-43; Clerici, M and Shearer, G.M. (1993) *Immunology Today* 14:107-111; Fauci, A.S. (1988) *Science* 239:617-623; Locksley, R. M. and Scott, P. (1992) *Immunoparasitology Today* 1:A58-A61; Pearce, E.J., *et al.* (1991) *J. Exp. Med.* 173:159-166; Grzych, J-M., *et al.* (1991) *J. Immunol.* 141:1322-1327; Kullberg, M.C., *et al.* (1992) *J. Immunol.* 148:3264-3270; Bancroft, A.J., *et al.* (1993) *J. Immunol.* 150:1395-1402; Pearlman, E.,

et al. (1993) *Infect. Immun.* 61:1105-1112; Else, K.J., *et al.* (1994) *J. Exp. Med.* 179:347-351) and such infectious diseases are also associated with a Th1 to Th2 shift in the immune response. Accordingly, the inhibitory methods of the invention can be used to inhibit the production of Th2-associated cytokines in subjects with infectious diseases, as a means to counteract the Th1 to Th2 shift and thereby promote an ongoing Th1 response in the patients to ameliorate the course of the infection. The inhibitory method can involve either direct administration of an inhibitory agent to a subject with an infectious disease or *ex vivo* treatment of cells obtained from the subject (*e.g.*, Thp or Th2 cells) with an inhibitory agent followed by readministration of the cells to the subject. The treatment may be further enhanced by administering other Th1-promoting agents, such as the cytokine IL-12 or antibodies to Th2-associated cytokines (*e.g.*, anti-IL-4 antibodies), to the recipient in amounts sufficient to further stimulate a Th1-type response.

D. *Autoimmune Diseases*

The stimulatory methods of the invention can be used therapeutically in the treatment of autoimmune diseases that are associated with a Th2-type dysfunction. Many autoimmune disorders are the result of inappropriate activation of T cells that are reactive against self tissue and that promote the production of cytokines and autoantibodies involved in the pathology of the diseases. Modulation of T helper-type responses can have an effect on the course of the autoimmune disease. For example, in experimental allergic encephalomyelitis (EAE), stimulation of a Th2-type response by administration of IL-4 at the time of the induction of the disease diminishes the intensity of the autoimmune disease (Paul, W.E., *et al.* (1994) *Cell* 76:241-251). Furthermore, recovery of the animals from the disease has been shown to be associated with an increase in a Th2-type response as evidenced by an increase of Th2-specific cytokines (Koury, S. J., *et al.* (1992) *J. Exp. Med.* 176:1355-1364). Moreover, T cells that can suppress EAE secrete Th2-specific cytokines (Chen, C., *et al.* (1994) *Immunity* 1:147-154). Since stimulation of a Th2-type response in EAE has a protective effect against the disease, stimulation of a Th2 response in subjects with multiple sclerosis (for which EAE is a model) is likely to be beneficial therapeutically.

Similarly, stimulation of a Th2-type response in type I diabetes in mice provides a protective effect against the disease. Indeed, treatment of NOD mice with IL-4 (which promotes a Th2 response) prevents or delays onset of type I diabetes that normally develops in these mice (Rapoport, M.J., *et al.* (1993) *J. Exp. Med.* 178:87-99). Thus,

stimulation of a Th2 response in a subject suffering from or susceptible to diabetes may ameliorate the effects of the disease or inhibit the onset of the disease.

Yet another autoimmune disease in which stimulation of a Th2-type response may be beneficial is rheumatoid arthritis (RA). Studies have shown that patients with rheumatoid arthritis have predominantly Th1 cells in synovial tissue (Simon, A.K., *et al.*, (1994) *Proc. Natl. Acad. Sci. USA* 91:8562-8566). By stimulating a Th2 response in a subject with RA, the detrimental Th1 response can be concomitantly downmodulated to thereby ameliorate the effects of the disease.

Accordingly, the stimulatory methods of the invention can be used to stimulate production of Th2-associated cytokines in subjects suffering from, or susceptible to, an autoimmune disease in which a Th2-type response is beneficial to the course of the disease. The stimulatory method can involve either direct administration of a stimulatory agent to the subject or *ex vivo* treatment of cells obtained from the subject (*e.g.*, Thp, Th1 cells, B cells, non-lymphoid cells) with a stimulatory agent followed by readministration of the cells to the subject. The treatment may be further enhanced by administering other Th2-promoting agents, such as IL-4 itself or antibodies to Th1-associated cytokines, to the subject in amounts sufficient to further stimulate a Th2-type response.

In contrast to the autoimmune diseases described above in which a Th2 response is desirable, other autoimmune diseases may be ameliorated by a Th1-type response. Such diseases can be treated using an inhibitory agent of the invention (as described above for cancer and infectious diseases). The treatment may be further enhanced by administrating a Th1-promoting cytokine (*e.g.*, IFN-γ) to the subject in amounts sufficient to further stimulate a Th1-type response.

The efficacy of agents for treating autoimmune diseases can be tested in the above described animal models of human diseases (*e.g.*, EAE as a model of multiple sclerosis and the NOD mice as a model for diabetes) or other well characterized animal models of human autoimmune diseases. Such animal models include the *mrl/lpr/lpr* mouse as a model for lupus erythematosus, murine collagen-induced arthritis as a model for rheumatoid arthritis, and murine experimental myasthenia gravis (see Paul ed., *Fundamental Immunology*, Raven Press, New York, 1989, pp. 840-856). A modulatory (*i.e.*, stimulatory or inhibitory) agent of the invention is administered to test animals and the course of the disease in the test animals is then monitored by the standard methods for the particular model being used. Effectiveness of the modulatory agent is evidenced by amelioration of the disease condition in animals treated with the agent as compared to untreated animals (or animals treated with a control agent).

Non-limiting examples of autoimmune diseases and disorders having an autoimmune component that may be treated according to the invention include diabetes mellitus, arthritis (including rheumatoid arthritis, juvenile rheumatoid arthritis, osteoarthritis, psoriatic arthritis), multiple sclerosis, myasthenia gravis, systemic lupus erythematosis, autoimmune thyroiditis, dermatitis (including atopic dermatitis and eczematous dermatitis), psoriasis, Sjögren's Syndrome, including keratoconjunctivitis sicca secondary to Sjögren's Syndrome, alopecia areata, allergic responses due to arthropod bite reactions, Crohn's disease, aphthous ulcer, iritis, conjunctivitis, keratoconjunctivitis, ulcerative colitis, asthma, allergic asthma, cutaneous lupus erythematosus, scleroderma, vaginitis, proctitis, drug eruptions, leprosy reversal reactions, erythema nodosum leprosum, autoimmune uveitis, allergic encephalomyelitis, acute necrotizing hemorrhagic encephalopathy, idiopathic bilateral progressive sensorineural hearing loss, aplastic anemia, pure red cell anemia, idiopathic thrombocytopenia, polychondritis, Wegener's granulomatosis, chronic active hepatitis, Stevens-Johnson syndrome, idiopathic sprue, lichen planus, Crohn's disease, Graves ophthalmopathy, sarcoidosis, primary biliary cirrhosis, uveitis posterior, and interstitial lung fibrosis.

E. *Transplantation*

While graft rejection or graft acceptance may not be attributable exclusively to the action of a particular T cell subset (*i.e.*, Th1 or Th2 cells) in the graft recipient (for a discussion see Dallman, M.J. (1995) *Curr. Opin. Immunol.* 7:632-638), numerous studies have implicated a predominant Th2 response in prolonged graft survival or a predominant Th2 response in graft rejection. For example, graft acceptance has been associated with production of a Th2 cytokine pattern and/or graft rejection has been associated with production of a Th1 cytokine pattern (see *e.g.*, Takeuchi, T. *et al.* (1992) *Transplantation* 53:1281-1291; Tzakis, A.G. *et al.* (1994) *J. Pediatr. Surg.* 29:754-756; Thai, N.L. *et al.* (1995) *Transplantation* 59:274-281). Additionally, adoptive transfer of cells having a Th2 cytokine phenotype prolongs skin graft survival (Maeda, H. *et al.* (1994) *Int. Immunol.* 6:855-862) and reduces graft-versus-host disease (Fowler, D.H. *et al.* (1994) *Blood* 84:3540-3549; Fowler, D.H. *et al.* (1994) *Prog. Clin. Biol. Res.* 389:533-540). Still further, administration of IL-4, which promotes Th2 differentiation, prolongs cardiac allograft survival (Levy, A.E. and Alexander, J.W. (1995) *Transplantation* 60:405-406), whereas administration of IL-12 in combination with anti-

IL-10 antibodies, which promotes Th1 differentiation, enhances skin allograft rejection (Gorczynski, R.M. *et al.* (1995) *Transplantation* 60:1337-1341).

Accordingly, the stimulatory methods of the invention can be used to stimulate production of Th2-associated cytokines in transplant recipients to prolong survival of the graft. The stimulatory methods can be used both in solid organ transplantation and in bone marrow transplantation (*e.g.*, to inhibit graft-versus-host disease). The stimulatory method can involve either direct administration of a stimulatory agent to the transplant recipient or *ex vivo* treatment of cells obtained from the subject (*e.g.*, Thp, Th1 cells, B cells, non-lymphoid cells) with a stimulatory agent followed by readministration of the cells to the subject. The treatment may be further enhanced by administering other Th2-promoting agents, such as IL-4 itself or antibodies to Th1-associated cytokines, to the recipient in amounts sufficient to further stimulate a Th2-type response.

In addition to the foregoing disease situations, the modulatory methods of the invention also are useful for other purposes. For example, the stimulatory methods of the invention (*i.e.*, methods using a stimulatory agent) can be used to stimulate production of Th2-promoting cytokines (*e.g.*, IL-4) *in vitro* for commercial production of these cytokines (*e.g.*, cells can be contacted with the stimulatory agent *in vitro* to stimulate IL-4 production and the IL-4 can be recovered from the culture supernatant, further purified if necessary, and packaged for commercial use).

Furthermore, the modulatory methods of the invention can be applied to vaccinations to promote either a Th1 or a Th2 response to an antigen of interest in a subject. That is, the agents of the invention can serve as adjuvants to direct an immune response to a vaccine either to a Th1 response or a Th2 response. For example, to stimulate an antibody response to an antigen of interest (*i.e.*, for vaccination purposes), the antigen and a stimulatory agent of the invention can be coadministered to a subject to promote a Th2 response to the antigen in the subject, since Th2 responses provide efficient B cell help and promote IgG1 production. Alternatively, to promote a cellular immune response to an antigen of interest, the antigen and an inhibitory agent of the invention can be coadministered to a subject to promote a Th1 response to the antigen in a subject, since Th1 responses favor the development of cell-mediated immune responses (*e.g.*, delayed hypersensitivity responses). The antigen of interest and the modulatory agent can be formulated together into a single pharmaceutical composition or in separate compositions. In a preferred embodiment, the antigen of interest and the modulatory agent are administered simultaneously to the subject. Alternatively, in

certain situations it may be desirable to administer the antigen first and then the modulatory agent or vice versa (for example, in the case of an antigen that naturally evokes a Th1 response, it may be beneficial to first administer the antigen alone to stimulate a Th1 response and then administer a stimulatory agent, alone or together with a boost of antigen, to shift the immune response to a Th2 response).

IV. Maf Compositions

Another aspect of the invention pertains to compositions that can be used to modulate Th2-associated cytokine production by a cell or Th1/Th2 development in a subject in accordance with the methods of the invention. The invention provides recombinant expression vectors comprising a nucleotide sequence encoding a maf family protein operatively linked to regulatory sequences that direct expression of the maf family protein specifically in certain cell types. In a preferred embodiment, the regulatory sequences direct expression of the maf family protein specifically in lymphoid cells (*e.g.*, T cells or B cells). In one embodiment, the lymphoid cells are T cells. T cell specific regulatory elements are known in the art, such as the promoter regulatory region of T cell receptor genes (see *e.g.*, Winoto and Baltimore (1989) *EMBO J.* 8:729-733; Leiden, J.M. (1994) *Annu. Rev. Immunol.* 11:539-570; Hettman, T. and Cohen, A. (1994) *Mol. Immunol.* 31:315-322; Redondo, J.M. *et al.* (1991) *Mol. Cell. Biol.* 11:5671-5680). Other examples of T cell specific regulatory elements are those derived from the CD3 gene (see *e.g.*, Clevers, H. *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:8623-8627; Clevers, H.C. *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:8156-8160; Georgopoulos, K. *et al.* (1988) *EMBO J.* 7:2401-2407), the CD4 gene (see *e.g.*, Sawada, S. and Littman, D.R. (1991) *Mol. Cell. Biol.* 11:5506-5515; Salmon, P. *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:7739-7743; Hanna, Z. *et al.* (1994) *Mol. Cell. Biol.* 14:1084-1094; see also GenBank accession numbers U01066 and S68043 for human CD4 promoter sequences) and the CD2 gene (see *e.g.*, Zhumabekov, T. *et al.* (1995) *J. Immunol. Methods* 185:133-140). A DNA fragment comprising one or more T cell specific regulatory elements, such as a promoter and enhancer of a T cell receptor gene, can be obtained by standard molecular biology methods, such as by PCR using oligonucleotide primers corresponding to the 5' and 3' ends of the desired region and genomic DNA from T cells as the template. Once the DNA fragment comprising T cell specific regulatory elements is obtained, it can be operatively linked to a cDNA encoding a maf protein (*e.g.*, the two DNA fragments can be ligated together such that the regulatory elements are located 5' of the maf sequences) and introduced into vector, such as a plasmid vector, using standard molecular biology techniques.

In another embodiment, the lymphoid cells are B cells (*i.e.*, within the recombinant expression vector the nucleotide sequences encoding a maf family protein are operatively linked to regulatory sequences that direct expression of the maf family specifically in B cells). B cell specific regulatory elements are known in the art, such as the promoter regulatory region of immunoglobulin genes (see *e.g.*, Banerji *et al.* (1983) *Cell* 33:729-740; Queen and Baltimore (1983) *Cell* 33:741-748). Other examples of B cell specific regulatory elements are those derived from the CD20 (B1) gene (see *e.g.*, Thevenin, C. *et al.* (1993) *J. Biol. Chem.* 268:5949-5956; Rieckmann, P. *et al.* (1991) *J. Immunol.* 147:3994-3999), the Fc epsilon RIIa gene (see *e.g.*, Suter, U. *et al.* (1989) *J. Immunol.* 143:3087-3092) and major histocompatibility class II genes (see *e.g.*, Glimcher, L.H. and Kara, C.J. (1992) *Annu. Rev. Immunol.* 10:13-49; Benoist, C. and Mathis, D. (1990) *Annu. Rev. Immunol.* 8:681-715). A DNA fragment comprising B cell specific regulatory elements, such as a promoter and enhancer of an immunoglobulin gene, can be obtained by standard molecular biology methods, such as by PCR using oligonucleotide primers corresponding to the 5' and 3' ends of the desired region and genomic DNA from B cells as the template. Once the DNA fragment comprising B cell specific regulatory elements is obtained, it can be operatively linked to a cDNA encoding a maf protein (*e.g.*, the two DNA fragments can be ligated together such that the regulatory elements are located 5' of the maf sequences) and introduced into vector, such as a plasmid vector, using standard molecular biology techniques.

In yet another embodiment, the invention provides recombinant expression vectors comprising a nucleotide sequence encoding a maf family protein operatively linked to regulatory sequences that direct expression of the maf family protein specifically in hematopoietic stem cells. Hematopoietic stem cell specific regulatory elements are known in the art. Preferably regulatory elements derived from the CD34 gene are used (see *e.g.*, Satterthwaite, A.B. *et al.* (1992) *Genomics* 12:788-794; Burn, T. C. *et al.* (1992) *Blood* 80:3051-3059).

Another aspect of the invention pertains to recombinant host cells that express a maf family protein. Such host cells can be used to produce a Th2-associated cytokine (*e.g.*, IL-4). Such host cells also can be administered to a subject to produce a Th2-associated cytokine in the subject as a means to manipulate Th1:Th2 ratios in the subject. The terms "host cell" and "recombinant host cell" are used interchangeably herein to refer to a cell into which a recombinant expression vector has been introduced. It is understood that such terms refer not only to the particular subject cell but to the progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not,

in fact, be identical to the parent cell, but as long as these progeny cells retain the recombinant expression vector, these progeny are still intended to be included within the scope of the term "host cell" as used herein.

In one embodiment, the invention provides a host lymphoid cell into which a recombinant expression vector encoding a maf family protein has been introduced. The host lymphoid cell can be a T cell or a B cell. A host T cell of the invention can be, for example a T cell clone that is cultured *in vitro* (such as those described in the Examples) or, alternatively, a normal T cell that is isolated from a subject (*e.g.*, a peripheral blood T cell or a splenic T cell). Standard methods for preparing and culturing T cell clones *in vitro*, or isolating T cells (*e.g.*, from peripheral blood) are known in the art, for example through the use of mAbs that bind T cell specific cell surface markers (*e.g.*, CD3) or surface markers for specific subsets of T cells (*e.g.*, CD4 or CD8). The recombinant expression vector can be introduced into the T cell by one of a variety of known transfection methods for introducing DNA into mammalian cells, including calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, or electroporation. Suitable methods for transforming or transfecting host cells can be found in Sambrook *et al.* (*Molecular Cloning: A Laboratory Manual*, 2nd Edition, Cold Spring Harbor Laboratory press (1989)), and other laboratory manuals.

In another embodiment, the host lymphoid cell of the invention is a host B cell into which a recombinant expression vector encoding a maf family protein has been introduced. The B cell can be, for example a B lymphoma cell that is cultured *in vitro* (such as M12 cells as described in the Examples) or, alternatively, a normal B cell that is isolated from a subject (*e.g.*, a peripheral blood B cell or a splenic B cell). Various B lymphoma cell lines are available in the art and standard methods for culturing such cells *in vitro* are known. Additionally, standard methods for isolating normal B cells (*e.g.*, from peripheral blood) are known in the art, for example through the use of mAbs that bind B cell specific cell surface markers (*e.g.*, membrane immunoglobulin, B7-1, CD20). The recombinant expression vector can be introduced into the B cell by standard methods, as described above for T cells.

In yet another embodiment, the invention provides a host hematopoietic stem cell into which a recombinant expression vector encoding a maf family protein has been introduced. Hematopoietic stem cells can be isolated from a subject (*e.g.*, from peripheral blood or bone marrow of the subject) using standard methods known in the art for isolating such stem cells, for example through the use of mAbs that bind hematopoietic stem cell specific cell surface markers, preferably CD34 (for further descriptions of isolation of stem cells, see *e.g.*, Wagner, J.E. *et al.* (1995) *Blood* 86:512-

523; Murray, L. *et al.* (1995) *Blood* 85:368-378; Bernardi, A.C. *et al.* (1995) *Science* 267:104-108; Bernstein, I.D. *et al.* (1994) *Blood Cells* 20:15-24; Angelini, A. *et al.* (1993) *Int. J. Artif. Organs* 16 Suppl. 5:13-18; Kato, K. and Radburch, A. (1993) *Cytometry* 14:384-392; Lebkowski, J.S. *et al.* (1992) *Transplantation* 53:1011-1019; Lebkowski, J. *et al.* (1993) *J. Hematother.* 2:339-342). The recombinant expression vector can be introduced into the hematopoietic stem cell by standard methods, as described above for T cells.

The skilled artisan will appreciate that the compositions described above with regard to maf family proteins can be prepared for various different maf family proteins, such as c-Maf and small mafs (*e.g.*, p18).

The invention further provides transgenic animals carrying a transgene encoding a maf family protein. In a preferred embodiment, the maf family transgene is expressed preferentially or exclusively in T cells of the animal. Tissue-specific expression of maf family proteins can be achieved through linkage of the maf coding sequences in the transgene to regulatory sequences that direct expression of the encoded protein in a particular cell type. Such tissue-specific regulatory elements are known in the art and are described further herein. A preferred regulatory element for T cell-specific expression of a maf family transgene is the CD4 promoter/enhancer. A preferred maf family protein for use in the transgene is c-maf. A schematic diagram of a preferred c-maf transgenic construct is shown in Figure 20. In this construct, the CD4 promoter/enhancer is operatively linked to the first intron of the mouse c-maf gene, which in turn is operatively linked to the mouse c-maf cDNA, which in turn is operatively linked to an SV40 polyadenylation sequence. Transgenic animals can be prepared by microinjecting a c-maf transgene construct into fertilized oocytes according to standard procedures for making transgenic animals, described in further detail herein. The phenotype of c-maf transgenic mice that overexpress c-Maf protein in T cells is described further in Example 17. In brief, these animals exhibit a phenotype very similar to IL-4 overexpressing transgenic mice, such as small spleen and thymus, decreased numbers of double positive thymocytes and single positive CD4+ thymocytes, and increased basal levels of serum IgE. Transgenic animals expressing a maf family protein can be used, for example, to evaluate test compounds for their ability to modulate the activity of the maf family protein. For example, a test compound can be administered to transgenic animal expressing a maf family protein and the effect of the test compound in the transgenic animal can be determined by comparing the phenotype of the transgenic animal in the presence of the test compound to the phenotype of the transgenic animal in the absence of the test compound.

V. NIP45 Compositions and Methods of Use Thereof

A. Isolated Nucleic Acid Molecules

One aspect of the invention pertains to isolated nucleic acid molecules that encode NIP45, or fragments thereof. In a preferred embodiment, an isolated nucleic acid molecule of the invention comprises the nucleotide sequence shown in SEQ ID NO: 5. The sequence of SEQ ID NO: 5 corresponds to the mouse NIP45 cDNA. This cDNA comprises sequences encoding the NIP45 protein (*i.e.*, "the coding region", from nucleotides 13-1248), as well as 5' untranslated sequences (nucleotides 1-12) and 3' untranslated sequences (nucleotides 1249-1946). Alternatively, the nucleic acid molecule may comprise only the coding region of SEQ ID NO: 5 (*i.e.*, nucleotides 13-1248).

Moreover, the nucleic acid molecule of the invention can comprise only a portion of the coding region of SEQ ID NO: 5, for example a fragment encoding a biologically active portion of NIP45. The term "biologically active portion of NIP45" is intended to include portions of NIP45 that retain the ability to interact with the RHD of NF-AT family proteins. The ability of portions of NIP45 to interact with an NF-AT RHD can be determined in standard *in vitro* interaction assays, for example using a NF-AT RHD fusion protein. Nucleic acid fragments encoding biologically active portions of NIP45 can be prepared by isolating a portion of SEQ ID NO: 5, expressing the encoded portion of NIP45 protein or peptide (*e.g.*, by recombinant expression in a host cell) and assessing the ability of the portion to interact with NF-AT, in particular the NF-AT RHD, for example using a glutathione-S-transferase (GST)-NF-AT RHD fusion protein.

The invention further encompasses nucleic acid molecules that differ from SEQ ID NO: 5 (and fragments thereof) due to degeneracy of the genetic code and thus encode the same NIP45 protein as that encoded by SEQ ID NO: 5. Accordingly, in another embodiment, an isolated nucleic acid molecule of the invention has a nucleotide sequence encoding a protein having an amino acid sequence shown in SEQ ID NO: 6. Moreover, the invention encompasses nucleic acid molecules that encode portions of SEQ ID NO: 6, such as biologically active portions thereof.

A nucleic acid molecule having the nucleotide sequence of SEQ ID NO: 5, or a portion thereof, can be isolated using standard molecular biology techniques and the sequence information provided herein. For example, a NIP45 cDNA can be isolated from a cDNA library using all or portion of SEQ ID NO: 5 as a hybridization probe and

standard hybridization techniques (*e.g.*, as described in Sambrook, J., *et al. Molecular Cloning: A Laboratory Manual. 2nd, ed., Cold Spring Harbor Laboratory,* Cold Spring Harbor, NY, 1989). Moreover, a nucleic acid molecule encompassing all or a portion of SEQ ID NO: 5 can be isolated by the polymerase chain reaction using oligonucleotide primers designed based upon the sequence of SEQ ID NO: 5. For example, mRNA can be isolated from cells (*e.g.*, by the guanidinium-thiocyanate extraction procedure of Chirgwin *et al.* (1979) *Biochemistry* 18: 5294-5299) and cDNA can be prepared using reverse transcriptase (*e.g.*, Moloney MLV reverse transcriptase, available from Gibco/BRL, Bethesda, MD; or AMV reverse transcriptase, available from Seikagaku America, Inc., St. Petersburg, FL). Synthetic oligonucleotide primers for PCR amplification can be designed based upon the nucleotide sequence shown in SEQ ID NO: 5. A nucleic acid of the invention can be amplified using cDNA or, alternatively, genomic DNA, as a template and appropriate oligonucleotide primers according to standard PCR amplification techniques. The nucleic acid so amplified can be cloned into an appropriate vector and characterized by DNA sequence analysis. Furthermore, oligonucleotides corresponding to a NIP45 nucleotide sequence can be prepared by standard synthetic techniques, *e.g.*, using an automated DNA synthesizer.

In addition to the NIP45 nucleotide sequence shown in SEQ ID NO: 5, it will be appreciated by those skilled in the art that DNA sequence polymorphisms that lead to changes in the amino acid sequences of NIP45 may exist within a population. Such genetic polymorphism in the NIP45 gene may exist among individuals within a population due to natural allelic variation. Such natural allelic variations can typically result in 1-5 % variance in the nucleotide sequence of the a gene. Any and all such nucleotide variations and resulting amino acid polymorphisms in NIP45 that are the result of natural allelic variation and that do not alter the functional activity of NIP45 are intended to be within the scope of the invention. Moreover, nucleic acid molecules encoding NIP45 proteins from other species, and thus which have a nucleotide sequence that differs from the mouse sequence of SEQ ID NO: 5 but that is related to the mouse sequence, are intended to be within the scope of the invention. Nucleic acid molecules corresponding to natural allelic variants and human and other mammalian homologues of the mouse NIP45 cDNA of the invention can be isolated based on their homology to the mouse NIP45 nucleic acid molecule disclosed herein using the mouse cDNA, or a portion thereof, as a hybridization probe according to standard hybridization techniques under stringent hybridization conditions. Accordingly, in another embodiment, an isolated nucleic acid molecule of the invention hybridizes under stringent conditions to the nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO: 5. In

certain embodiment, the nucleic acid is at least 15, 30, 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 or 1500 nucleotides in length. Preferably, an isolated nucleic acid molecule of the invention that hybridizes under stringent conditions to the sequence of SEQ ID NO: 5 corresponds to a naturally-occurring nucleic acid molecule. In on embodiment, the nucleic acid encodes natural human NIP45 protein. In another embodiment, the nucleic acid molecule encodes a murine NIP45 protein, such as mouse NIP45 protein.

In addition to naturally-occurring allelic variants of the NIP45 sequence that may exist in the population, the skilled artisan will further appreciate that changes may be introduced by mutation into the nucleotide sequence of SEQ ID NO: 5, thereby leading to changes in the amino acid sequence of the encoded protein, without altering the functional activity of the NIP45 protein. For example, nucleotide substitutions leading to amino acid substitutions at "non-essential" amino acid residues may be made in the sequence of SEQ ID NO: 5. A "non-essential" amino acid residue is a residue that can be altered from the wild-type sequence of NIP45 (*e.g.*, the sequence of SEQ ID NO: 6) without altering the functional activity of NIP45, such as its ability to interact with an NF-AT RHD or its ability to synergize with NF-AT and c-Maf in stimulating gene transcription, whereas an "essential" amino acid residue is required for functional activity.

Accordingly, another aspect of the invention pertains to nucleic acid molecules encoding NIP45 proteins that contain changes in amino acid residues that are not essential for NIP45 activity. Such NIP45 proteins differ in amino acid sequence from SEQ ID NO: 6 yet retain NIP45 activity. In one embodiment, the isolated nucleic acid molecule comprises a nucleotide sequence encoding a protein, wherein the protein comprises an amino acid sequence at least 60 % homologous to the amino acid sequence of SEQ ID NO: 6 and interacts with the RHD of an NF-AT family protein. Preferably, the protein encoded by the nucleic acid molecule is at least 70 % homologous to SEQ ID NO: 6, more preferably at least 80 % homologous to SEQ ID NO: 6, even more preferably at least 90 % homologous to SEQ ID NO: 6, and most preferably at least 95 % homologous to SEQ ID NO: 6.

To determine the percent homology of two amino acid sequences (*e.g.*, SEQ ID NO: 6 and a mutant form thereof), the sequences are aligned for optimal comparison purposes (*e.g.*, gaps may be introduced in the sequence of one protein for optimal alignment with the other protein). The amino acid residues at corresponding amino acid positions are then compared. When a position in one sequence (*e.g.*, SEQ ID NO: 6) is occupied by the same amino acid residue as the corresponding position in the other

sequence (*e.g.*, a mutant form of NIP45), then the molecules are homologous at that position (*i.e.*, as used herein amino acid "homology" is equivalent to amino acid "identity"). The percent homology between the two sequences is a function of the number of identical positions shared by the sequences (*i.e.*, % homology = # of identical positions/total # of positions x 100).

An isolated nucleic acid molecule encoding a NIP45 protein homologous to the protein of SEQ ID NO: 6 can be created by introducing one or more nucleotide substitutions, additions or deletions into the nucleotide sequence of SEQ ID NO: 5 such that one or more amino acid substitutions, additions or deletions are introduced into the encoded protein. Mutations can be introduced into SEQ ID NO: 5 by standard techniques, such as site-directed mutagenesis and PCR-mediated mutagenesis. Preferably, conservative amino acid substitutions are made at one or more predicted non-essential amino acid residues. A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art, including basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (*e.g.*, threonine, valine, isoleucine) and aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan, histidine). Thus, an amino acid residue in NIP45 protein is preferably replaced with another amino acid residue from the same side chain family. Alternatively, in another embodiment, mutations can be introduced randomly along all or part of a NIP45 coding sequence, such as by saturation mutagenesis, and the resultant mutants can be screened for their ability to interact with an NF-AT RHD (*e.g.*, using a GST-NF-AT-RHD fusion protein) to identify mutants that retain NF-AT-interacting ability. Following mutagenesis of SEQ ID NO: 5, the encoded mutant protein can be expressed recombinantly in a host cell and the ability of the mutant protein to interact with NF-AT can be determined using an *in vitro* interaction assay. For example, a recombinant NIP45 protein (*e.g.*, a mutated or truncated form of SEQ ID NO: 6) can be radiolabeled and incubated with a GST-NF-AT RHD fusion protein. Glutathione-sepharose beads are then added to the mixture to precipitate the NIP45-GST-NF-AT RHD complex, if such a complex is formed. After washing the beads to remove non-specific binding, the amount of radioactive protein associated with the beads is determined and compared to the amount of radioactive protein remaining in

the eluate to thereby determine whether the NIP45 protein is capable of interacting with the RHD of NF-AT.

Another aspect of the invention pertains to isolated nucleic acid molecules that are antisense to the coding strand of a NIP45 mRNA or gene. An antisense nucleic acid of the invention can be complementary to an entire NIP45 coding strand, or to only a portion thereof. In one embodiment, an antisense nucleic acid molecule is antisense to a coding region of the coding strand of a nucleotide sequence encoding NIP45 (*e.g.*, the entire coding region of SEQ ID NO: 5 comprises nucleotides 13-1248). In another embodiment, the antisense nucleic acid molecule is antisense to a noncoding region of the coding strand of a nucleotide sequence encoding NIP45. In certain embodiments, an antisense nucleic acid of the invention is at least 15, 30, 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 or 1500 nucleotides in length.

Given the coding strand sequences encoding NIP45 disclosed herein (*e.g.*, SEQ ID NO: 5), antisense nucleic acids of the invention can be designed according to the rules of Watson and Crick base pairing. The antisense nucleic acid molecule may be complementary to the entire coding region of NIP45 mRNA, or alternatively can be an oligonucleotide which is antisense to only a portion of the coding or noncoding region of NIP45 mRNA. For example, the antisense oligonucleotide may be complementary to the region surrounding the translation start site of NIP45 mRNA. An antisense oligonucleotide can be, for example, about 15, 20, 25, 30, 35, 40, 45 or 50 nucleotides in length. An antisense nucleic acid of the invention can be constructed using chemical synthesis and enzymatic ligation reactions using procedures known in the art. For example, an antisense nucleic acid (*e.g.*, an antisense oligonucleotide) can be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed between the antisense and sense nucleic acids, *e.g.*, phosphorothioate derivatives and acridine substituted nucleotides can be used. Alternatively, the antisense nucleic acid can be produced biologically using an expression vector into which a nucleic acid has been subcloned in an antisense orientation (*i.e.*, RNA transcribed from the inserted nucleic acid will be of an antisense orientation to a target nucleic acid of interest, described further in the following subsection).

In another embodiment, an antisense nucleic acid of the invention is a ribozyme. Ribozymes are catalytic RNA molecules with ribonuclease activity which are capable of cleaving a single-stranded nucleic acid, such as an mRNA, to which they have a complementary region. A ribozyme having specificity for a NIP45-encoding nucleic

acid can be designed based upon the nucleotide sequence of a NIP45 cDNA disclosed herein (*i.e.*, SEQ ID NO: 5). For example, a derivative of a *Tetrahymena* L-19 IVS RNA can be constructed in which the base sequence of the active site is complementary to the base sequence to be cleaved in a NIP45-encoding mRNA. See for example Cech *et al.* U.S. Patent No. 4,987,071; and Cech *et al.* U.S. Patent No. 5,116,742. Alternatively, NIP45 mRNA can be used to select a catalytic RNA having a specific ribonuclease activity from a pool of RNA molecules. See for example Bartel, D. and Szostak, J.W. (1993) *Science* 261: 1411-1418.

Yet another aspect of the invention pertains to isolated nucleic acid molecules encoding NIP45 fusion proteins. Such nucleic acid molecules, comprising at least a first nucleotide sequence encoding a NIP45 protein, polypeptide or peptide operatively linked to a second nucleotide sequence encoding a non-NIP45 protein, polypeptide or peptide, can be prepared by standard recombinant DNA techniques. NIP45 fusion proteins are described in further detail below in subsection C.

B. Recombinant Expression Vectors and Host Cells

Another aspect of the invention pertains to vectors, preferably recombinant expression vectors, containing a nucleic acid encoding NIP45 (or a portion thereof). The expression vectors of the invention comprise a nucleic acid of the invention in a form suitable for expression of the nucleic acid in a host cell, which means that the recombinant expression vectors include one or more regulatory sequences, selected on the basis of the host cells to be used for expression, which is operatively linked to the nucleic acid sequence to be expressed. Within a recombinant expression vector, "operably linked" is intended to mean that the nucleotide sequence of interest is linked to the regulatory sequence(s) in a manner which allows for expression of the nucleotide sequence (*e.g.*, in an *in vitro* transcription/translation system or in a host cell when the vector is introduced into the host cell). The term "regulatory sequence" is intended to includes promoters, enhancers and other expression control elements (*e.g.*, polyadenylation signals). Such regulatory sequences are described, for example, in Goeddel; *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990). Regulatory sequences include those which direct constitutive expression of a nucleotide sequence in many types of host cell and those which direct expression of the nucleotide sequence only in certain host cells (*e.g.*, tissue-specific regulatory sequences). It will be appreciated by those skilled in the art that the design of the expression vector may depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, *etc.* The expression vectors of

the invention can be introduced into host cells to thereby produce proteins or peptides, including fusion proteins or peptides, encoded by nucleic acids as described herein (*e.g.*, NIP45 proteins, mutant forms of NIP45 proteins, NIP45 fusion proteins and the like).

The recombinant expression vectors of the invention can be designed for expression of NIP45 protein in prokaryotic or eukaryotic cells. For example, NIP45 can be expressed in bacterial cells such as *E. coli*, insect cells (using baculovirus expression vectors) yeast cells or mammalian cells. Suitable host cells are discussed further in Goeddel, *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990). Alternatively, the recombinant expression vector may be transcribed and translated *in vitro*, for example using T7 promoter regulatory sequences and T7 polymerase.

Expression of proteins in prokaryotes is most often carried out in *E. coli* with vectors containing constitutive or inducible promotors directing the expression of either fusion or non-fusion proteins. Fusion vectors add a number of amino acids to a protein encoded therein, usually to the amino terminus of the recombinant protein. Such fusion vectors typically serve three purposes: 1) to increase expression of recombinant protein; 2) to increase the solubility of the recombinant protein; and 3) to aid in the purification of the recombinant protein by acting as a ligand in affinity purification. Often, in fusion expression vectors, a proteolytic cleavage site is introduced at the junction of the fusion moiety and the recombinant protein to enable separation of the recombinant protein from the fusion moiety subsequent to purification of the fusion protein. Such enzymes, and their cognate recognition sequences, include Factor Xa, thrombin and enterokinase. Typical fusion expression vectors include pGEX (Pharmacia Biotech Inc; Smith, D.B. and Johnson, K.S. (1988) *Gene* 67:31-40), pMAL (New England Biolabs, Beverly, MA) and pRIT5 (Pharmacia, Piscataway, NJ) which fuse glutathione S-transferase (GST), maltose E binding protein, or protein A, respectively, to the target recombinant protein.

Examples of suitable inducible non-fusion *E. coli* expression vectors include pTrc (Amann *et al.*, (1988) *Gene* 69:301-315) and pET 11d (Studier *et al.*, *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, California (1990) 60-89). Target gene expression from the pTrc vector relies on host RNA polymerase transcription from a hybrid trp-lac fusion promoter. Target gene expression from the pET 11d vector relies on transcription from a T7 gn10-lac fusion promoter mediated by a coexpressed viral RNA polymerase (T7 gn1). This viral polymerase is supplied by host strains BL21(DE3) or HMS174(DE3) from a resident λ prophage harboring a T7 gn1 gene under the transcriptional control of the lacUV 5 promoter.

One strategy to maximize recombinant protein expression in *E. coli* is to express the protein in a host bacteria with an impaired capacity to proteolytically cleave the recombinant protein (Gottesman, S., *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, California (1990) 119-128). Another strategy is to alter the nucleic acid sequence of the nucleic acid to be inserted into an expression vector so that the individual codons for each amino acid are those preferentially utilized in *E. coli* (Wada *et al.*, (1992) *Nuc. Acids Res.* 20:2111-2118). Such alteration of nucleic acid sequences of the invention can be carried out by standard DNA synthesis techniques.

In another embodiment, the NIP45 expression vector is a yeast expression vector. Examples of vectors for expression in yeast *S. cerivisae* include pYepSec1 (Baldari. *et al.*, (1987) *EMBO J.* 6:229-234), pMFa (Kurjan and Herskowitz, (1982) *Cell* 30:933-943), pJRY88 (Schultz *et al.*, (1987) *Gene* 54:113-123), and pYES2 (Invitrogen Corporation, San Diego, CA).

Alternatively, NIP45 can be expressed in insect cells using baculovirus expression vectors. Baculovirus vectors available for expression of proteins in cultured insect cells (*e.g.*, Sf 9 cells) include the pAc series (Smith *et al.*, (1983) *Mol. Cell Biol.* 3:2156-2165) and the pVL series (Lucklow, V.A., and Summers, M.D., (1989) *Virology* 170:31-39).

In yet another embodiment, a nucleic acid of the invention is expressed in mammalian cells using a mammalian expression vector. Examples of mammalian expression vectors include pMex-NeoI, pCDM8 (Seed, B., (1987) *Nature* 329:840) and pMT2PC (Kaufman *et al.* (1987), *EMBO J.* 6:187-195). When used in mammalian cells, the expression vector's control functions are often provided by viral regulatory elements. For example, commonly used promoters are derived from polyoma, Adenovirus 2, cytomegalovirus and Simian Virus 40.

In another embodiment, the recombinant mammalian expression vector is capable of directing expression of the nucleic acid preferentially in a particular cell type (*e.g.*, tissue-specific regulatory elements are used to express the nucleic acid). Tissue-specific regulatory elements are known in the art. Non-limiting examples of suitable tissue-specific promoters include lymphoid-specific promoters (Calame and Eaton (1988) *Adv. Immunol.* 43:235-275), in particular promoters of T cell receptors (Winoto and Baltimore (1989) *EMBO J.* 8:729-733) and immunoglobulins (Banerji *et al.* (1983) *Cell* 33:729-740; Queen and Baltimore (1983) *Cell* 33:741-748), the albumin promoter (liver-specific; Pinkert *et al.* (1987) *Genes Dev.* 1:268-277), neuron-specific promoters (*e.g.*, the neurofilament promoter; Byrne and Ruddle (1989) *Proc. Natl. Acad. Sci. USA*

86:5473-5477), pancreas-specific promoters (Edlund *et al.* (1985) *Science* 230:912-916), and mammary gland-specific promoters (*e.g.*, milk whey promoter; U.S. Patent No. 4,873,316 and European Application Publication No. 264,166). Developmentally-regulated promoters are also encompassed, for example the murine hox promoters (Kessel and Gruss (1990) *Science* 249:374-379) and the α-fetoprotein promoter (Campes and Tilghman (1989) *Genes Dev.* 3:537-546).

The invention further provides a recombinant expression vector comprising a DNA molecule of the invention cloned into the expression vector in an antisense orientation. That is, the DNA molecule is operatively linked to a regulatory sequence in a manner which allows for expression (by transcription of the DNA molecule) of an RNA molecule which is antisense to NIP45 mRNA. Regulatory sequences operatively linked to a nucleic acid cloned in the antisense orientation can be chosen which direct the continuous expression of the antisense RNA molecule in a variety of cell types, for instance viral promoters and/or enhancers, or regulatory sequences can be chosen which direct constitutive, tissue specific or cell type specific expression of antisense RNA. The antisense expression vector can be in the form of a recombinant plasmid, phagemid or attenuated virus in which antisense nucleic acids are produced under the control of a high efficiency regulatory region, the activity of which can be determined by the cell type into which the vector is introduced. For a discussion of the regulation of gene expression using antisense genes see Weintraub, H. *et al.*, Antisense RNA as a molecular tool for genetic analysis, *Reviews - Trends in Genetics*, Vol. 1(1) 1986.

Another aspect of the invention pertains to recombinant host cells into which a vector, preferably a recombinant expression vector, of the invention has been introduced. A host cell may be any prokaryotic or eukaryotic cell. For example, NIP45 protein may be expressed in bacterial cells such as *E. coli*, insect cells, yeast or mammalian cells (such as Chinese hamster ovary cells (CHO) or COS cells). Other suitable host cells are known to those skilled in the art. Vector DNA can be introduced into prokaryotic or eukaryotic cells via conventional transformation or transfection techniques. As used herein, the terms "transformation" and "transfection" are intended to refer to a variety of art-recognized techniques for introducing foreign nucleic acid (*e.g.*, DNA) into a host cell, including calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, or electroporation. Suitable methods for transforming or transfecting host cells can be found in Sambrook *et al.* (*Molecular Cloning: A Laboratory Manual*, 2nd Edition, Cold Spring Harbor Laboratory press (1989)), and other laboratory manuals.

For stable transfection of mammalian cells, it is known that, depending upon the expression vector and transfection technique used, only a small fraction of cells may integrate the foreign DNA into their genome. In order to identify and select these integrants, a gene that encodes a selectable marker (*e.g.*, resistance to antibiotics) is generally introduced into the host cells along with the gene of interest. Preferred selectable markers include those which confer resistance to drugs, such as G418, hygromycin and methotrexate. Nucleic acid encoding a selectable marker may be introduced into a host cell on the same vector as that encoding NIP45 or may be introduced on a separate vector. Cells stably transfected with the introduced nucleic acid can be identified by drug selection (*e.g.*, cells that have incorporated the selectable marker gene will survive, while the other cells die).

A host cell of the invention, such as a prokaryotic or eukaryotic host cell in culture, can be used to produce (*i.e.*, express) NIP45 protein. Accordingly, the invention further provides methods for producing NIP45 protein using the host cells of the invention. In one embodiment, the method comprises culturing the host cell of invention (into which a recombinant expression vector encoding NIP45 has been introduced) in a suitable medium until NIP45 is produced. In another embodiment, the method further comprises isolating NIP45 from the medium or the host cell. In its native form NIP45 protein is an intracellular protein and, accordingly, recombinant NIP45 protein can be expressed intracellularly in a recombinant host cell and then isolated from the host cell, *e.g.*, by lysing the host cell and recovering the recombinant NIP45 protein from the lysate. Alternatively, recombinant NIP45 protein can be prepared as a extracellular protein by operatively linking a heterologous signal sequence to the amino-terminus of the protein such that the protein is secreted from the host cells. In this case, recombinant NIP45 protein can be recovered from the culture medium in which the cells are cultured.

Certain host cells of the invention can also be used to produce nonhuman transgenic animals. For example, in one embodiment, a host cell of the invention is a fertilized oocyte or an embryonic stem cell into which NIP45-coding sequences have been introduced. Such host cells can then be used to create non-human transgenic animals in which exogenous NIP45 sequences have been introduced into their genome or homologous recombinant animals in which endogenous NIP45 sequences have been altered. Such animals are useful for studying the function and/or activity of NIP45 and for identifying and/or evaluating modulators of NIP45 activity. Accordingly, another aspect of the invention pertains to nonhuman transgenic animals which contain cells carrying a transgene encoding a NIP45 protein or a portion of a NIP45 protein. In a

subembodiment, of the transgenic animals of the invention, the transgene alters an endogenous gene encoding an endogenous NIP45 protein (*e.g.*, homologous recombinant animals in which the endogenous NIP45 gene has been functionally disrupted or "knocked out", or the nucleotide sequence of the endogenous NIP45 gene has been mutated or the transcriptional regulatory region of the endogenous NIP45 gene has been altered).

A transgenic animal of the invention can be created by introducing NIP45-encoding nucleic acid into the male pronuclei of a fertilized oocyte, *e.g.*, by microinjection, and allowing the oocyte to develop in a pseudopregnant female foster animal. The mouse NIP45 cDNA sequence of SEQ ID NO: 5 can be introduced as a transgene into the genome of a non-human animal (*e.g.*, a mouse). Alternatively, a mammalian homologue of the mouse NIP45 gene, such as a human NIP45 gene, can be isolated based on hybridization to the mouse NIP45 cDNA and used as a transgene. Intronic sequences and polyadenylation signals can also be included in the transgene to increase the efficiency of expression of the transgene. A tissue-specific regulatory sequence(s) can be operably linked to the NIP45 transgene to direct expression of NIP45 protein to particular cells. Methods for generating transgenic animals via embryo manipulation and microinjection, particularly animals such as mice, have become conventional in the art and are described, for example, in U.S. Patent Nos. 4,736,866 and 4,870,009, both by Leder *et al.*, U.S. Patent No. 4,873,191 by Wagner *et al.* and in Hogan, B., *Manipulating the Mouse Embryo*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986). Similar methods are used for production of other transgenic animals. A transgenic founder animal can be identified based upon the presence of the NIP45 transgene in its genome and/or expression of NIP45 mRNA in tissues or cells of the animals. A transgenic founder animal can then be used to breed additional animals carrying the transgene. Moreover, transgenic animals carrying a transgene encoding NIP45 can further be bred to other transgenic animals carrying other transgenes.

To create a homologous recombinant animal, a vector is prepared which contains at least a portion of a NIP45 gene into which a deletion, addition or substitution has been introduced to thereby alter, *e.g.*, functionally disrupt, the endogenous NIP45 gene. The NIP45 gene preferably is a mouse NIP45 gene. For example, a mouse NIP45 gene can be isolated from a mouse genomic DNA library using the mouse NIP45 cDNA of SEQ ID NO: 5 as a probe. The mouse NIP45 gene then can be used to construct a homologous recombination vector suitable for altering an endogenous NIP45 gene in the mouse genome. In a preferred embodiment, the vector is designed such that, upon

homologous recombination, the endogenous NIP45 gene is functionally disrupted (*i.e.*, no longer encodes a functional protein; also referred to as a "knock out" vector). Alternatively, the vector can be designed such that, upon homologous recombination, the endogenous NIP45 gene is mutated or otherwise altered but still encodes functional protein (*e.g.*, the upstream regulatory region can be altered to thereby alter the expression of the endogenous NIP45 protein). In the homologous recombination vector, the altered portion of the NIP45 gene is flanked at its 5' and 3' ends by additional nucleic acid of the NIP45 gene to allow for homologous recombination to occur between the exogenous NIP45 gene carried by the vector and an endogenous NIP45 gene in an embryonic stem cell. The additional flanking NIP45 nucleic acid is of sufficient length for successful homologous recombination with the endogenous gene. Typically, several kilobases of flanking DNA (both at the 5' and 3' ends) are included in the vector (see *e.g.*, Thomas, K.R. and Capecchi, M. R. (1987) *Cell* 51:503 for a description of homologous recombination vectors). The vector is introduced into an embryonic stem cell line (*e.g.*, by electroporation) and cells in which the introduced NIP45 gene has homologously recombined with the endogenous NIP45 gene are selected (see *e.g.*, Li, E. *et al.* (1992) *Cell* 69:915). The selected cells are then injected into a blastocyst of an animal (*e.g.*, a mouse) to form aggregation chimeras (see *e.g.*, Bradley, A. in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, E.J. Robertson, ed. (IRL, Oxford, 1987) pp. 113-152). A chimeric embryo can then be implanted into a suitable pseudopregnant female foster animal and the embryo brought to term. Progeny harboring the homologously recombined DNA in their germ cells can be used to breed animals in which all cells of the animal contain the homologously recombined DNA by germline transmission of the transgene. Methods for constructing homologous recombination vectors and homologous recombinant animals are described further in Bradley, A. (1991) *Current Opinion in Biotechnology* 2:823-829 and in PCT International Publication Nos.: WO 90/11354 by Le Mouellec *et al.*; WO 91/01140 by Smithies *et al.*; WO 92/0968 by Zijlstra *et al.*; and WO 93/04169 by Berns *et al.*

C. Isolated NIP45 Proteins and Anti-NIP45 Antibodies

Another aspect of the invention pertains to isolated NIP45 proteins, and portions thereof, such as biologically active portions, as well as peptide fragments suitable as immunogens to raise anti-NIP45 antibodies. In one embodiment, the invention provides an isolated preparation of NIP45 protein. Preferably, the NIP45 protein has an amino acid sequence shown in SEQ ID NO: 6. In other embodiments, the NIP45 protein is substantially homologous to SEQ ID NO: 6 and retains the functional activity of the

protein of SEQ ID NO: 6 yet differs in amino acid sequence due to natural allelic variation or mutagenesis, or is a mammalian homologue of the protein of SEQ ID NO: 6 (e.g., a human homologue), as described in detail in subsection A above. Accordingly, in another embodiment, the NIP45 protein is a protein which comprises an amino acid sequence at least 60 % homologous to the amino acid sequence of SEQ ID NO: 6 and that interacts with the RHD of an NF-AT family protein. Preferably, the protein is at least 70 % homologous to SEQ ID NO: 6, more preferably at least 80 % homologous to SEQ ID NO: 6, even more preferably at least 90 % homologous to SEQ ID NO: 6, and most preferably at least 95 % homologous to SEQ ID NO: 6.

In other embodiments, the invention provides isolated portions of the NIP45 protein. For example, the invention further encompasses a portion of a NIP45 protein that interacts with NF-AT. As demonstrated in the examples, NIP45 protein interacts with the RHD of NF-AT. An *in vitro* interaction assay (such as that described above in subsection A utilizing a GST-NF-AT RHD fusion protein) can be used to determine the ability of NIP45 peptide fragments to interact with the NF-AT Rel Homology Domain to thereby identify peptide fragments that interact with NF-AT.

NIP45 proteins are preferably produced by recombinant DNA techniques. For example, a nucleic acid molecule encoding the protein is cloned into an expression vector (as described above), the expression vector is introduced into a host cell (as described above) and the NIP45 protein is expressed in the host cell. The NIP45 protein can then be isolated from the cells by an appropriate purification scheme using standard protein purification techniques. Alternative to recombinant expression, a NIP45 polypeptide can be synthesized chemically using standard peptide synthesis techniques. Moreover, native NIP45 protein can be isolated from cells (*e.g.*, from T cells), for example by immunoprecipitation using an anti-NIP45 antibody.

The invention also provides NIP45 fusion proteins. As used herein, a NIP45 "fusion protein" comprises a NIP45 polypeptide operatively linked to a non-NIP45 polypeptide. A "NIP45 polypeptide" refers to a polypeptide having an amino acid sequence corresponding to NIP45 protein, or a peptide fragment thereof, whereas a "non-NIP45 polypeptide" refers to a polypeptide having an amino acid sequence corresponding to another protein. Within the fusion protein, the term "operatively linked" is intended to indicate that the NIP45 polypeptide and the non-NIP45 polypeptide are fused in-frame to each other. The non-NIP45 polypeptide may be fused to the N-terminus or C-terminus of the NIP45 polypeptide. For example, in one embodiment, the fusion protein is a GST-NIP45 fusion protein in which the NIP45 sequences are fused to the C-terminus of the GST sequences. In another embodiment,

the fusion protein is a NIP45-HA fusion protein in which the NIP45 nucleotide sequence is inserted in to the pCEP4-HA vector (Herrscher, R.F. *et al.* (1995) *Genes Dev.* 9:3067-3082) such that the NIP45 sequences are fused in frame to an influenza hemagglutinin epitope tag. Such fusion proteins can facilitate the purification of recombinant NIP45.

Preferably, a NIP45 fusion protein of the invention is produced by standard recombinant DNA techniques. For example, DNA fragments coding for the different polypeptide sequences are ligated together in-frame in accordance with conventional techniques, for example employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments can be carried out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments which can subsequently be annealed and reamplified to generate a chimeric gene sequence (see, for example, *Current Protocols in Molecular Biology*, eds. Ausubel *et al.* John Wiley & Sons: 1992). Moreover, many expression vectors are commercially available that already encode a fusion moiety (*e.g.*, a GST polypeptide or an HA epitope tag). A NIP45-encoding nucleic acid can be cloned into such an expression vector such that the fusion moiety is linked in-frame to the NIP45 protein.

An isolated NIP45 protein, or fragment thereof, can be used as an immunogen to generate antibodies that bind NIP45 using standard techniques for polyclonal and monoclonal antibody preparation. The NIP45 protein can be used to generate antibodies or, alternatively, an antigenic peptide fragment of NIP45 can be used as the immunogen. An antigenic peptide fragment of NIP45 typically comprises at least 8 amino acid residues of the amino acid sequence shown in SEQ ID NO: 6 and encompasses an epitope of NIP45 such that an antibody raised against the peptide forms a specific immune complex with NIP45. Preferably, the antigenic peptide comprises at least 10 amino acid residues, more preferably at least 15 amino acid residues, even more preferably at least 20 amino acid residues, and most preferably at least 30 amino acid residues. Preferred epitopes encompassed by the antigenic peptide are regions of NIP45 that are located on the surface of the protein, *e.g.*, hydrophilic regions. A hydrophobicity analysis of the NIP45 protein sequence of SEQ ID NO: 6 is shown in Figure 12.

A NIP45 immunogen typically is used to prepare antibodies by immunizing a suitable subject, (*e.g.*, rabbit, goat, mouse or other mammal) with the immunogen. An

appropriate immunogenic preparation can contain, for examples, recombinantly expressed NIP45 protein or a chemically synthesized NIP45 peptide. The preparation can further include an adjuvant, such as Freund's complete or incomplete adjuvant, or similar immunostimulatory agent. Immunization of a suitable subject with an immunogenic NIP45 preparation induces a polyclonal anti-NIP45 antibody response.

Accordingly, another aspect of the invention pertains to anti-NIP45 antibodies. Polyclonal anti-NIP45 antibodies can be prepared as described above by immunizing a suitable subject with a NIP45 immunogen. The anti-NIP45 antibody titer in the immunized subject can be monitored over time by standard techniques, such as with an enzyme linked immunosorbent assay (ELISA) using immobilized NIP45. If desired, the antibody molecules directed against NIP45 can be isolated from the mammal (*e.g.*, from the blood) and further purified by well known techniques, such as protein A chromatography to obtain the IgG fraction. At an appropriate time after immunization, *e.g.*, when the anti-NIP45 antibody titers are highest, antibody-producing cells can be obtained from the subject and used to prepare monoclonal antibodies by standard techniques, such as the hybridoma technique originally described by Kohler and Milstein (1975, *Nature* 256:495-497) (see also, Brown *et al.* (1981) *J. Immunol* 127:539-46; Brown *et al.* (1980) *J Biol Chem* 255:4980-83; Yeh *et al.* (1976) *PNAS* 76:2927-31; and Yeh *et al.* (1982) *Int. J. Cancer* 29:269-75), the more recent human B cell hybridoma technique (Kozbor *et al.* (1983) *Immunol Today* 4:72), the EBV-hybridoma technique (Cole *et al.* (1985), *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96) or trioma techniques. The technology for producing monoclonal antibody hybridomas is well known (see generally R. H. Kenneth, in *Monoclonal Antibodies: A New Dimension In Biological Analyses*, Plenum Publishing Corp., New York, New York (1980); E. A. Lerner (1981) *Yale J. Biol. Med.*, 54:387-402; M. L. Gefter *et al.* (1977) *Somatic Cell Genet.*, 3:231-36). Briefly, an immortal cell line (typically a myeloma) is fused to lymphocytes (typically splenocytes) from a mammal immunized with a NIP45 immunogen as described above, and the culture supernatants of the resulting hybridoma cells are screened to identify a hybridoma producing a monoclonal antibody that binds NIP45.

Any of the many well known protocols used for fusing lymphocytes and immortalized cell lines can be applied for the purpose of generating an anti-NIP45 monoclonal antibody (see, *e.g.*, G. Galfre *et al.* (1977) *Nature* 266:55052; Gefter *et al. Somatic Cell Genet.*, cited *supra*; Lerner, *Yale J. Biol. Med.*, cited *supra*; Kenneth, *Monoclonal Antibodies*, cited *supra*). Moreover, the ordinary skilled worker will appreciate that there are many variations of such methods which also would be useful.

Typically, the immortal cell line (*e.g.*, a myeloma cell line) is derived from the same mammalian species as the lymphocytes. For example, murine hybridomas can be made by fusing lymphocytes from a mouse immunized with an immunogenic preparation of the present invention with an immortalized mouse cell line. Preferred immortal cell lines are mouse myeloma cell lines that are sensitive to culture medium containing hypoxanthine, aminopterin and thymidine ("HAT medium"). Any of a number of myeloma cell lines may be used as a fusion partner according to standard techniques, *e.g.*, the P3-NS1/1-Ag4-1, P3-x63-Ag8.653 or Sp2/O-Ag14 myeloma lines. These myeloma lines are available from the American Type Culture Collection (ATCC), Rockville, Md. Typically, HAT-sensitive mouse myeloma cells are fused to mouse splenocytes using polyethylene glycol ("PEG"). Hybridoma cells resulting from the fusion are then selected using HAT medium, which kills unfused and unproductively fused myeloma cells (unfused splenocytes die after several days because they are not transformed). Hybridoma cells producing a monoclonal antibody of the invention are detected by screening the hybridoma culture supernatants for antibodies that bind NIP45, *e.g.*, using a standard ELISA assay.

Alternative to preparing monoclonal antibody-secreting hybridomas, a monoclonal anti-NIP45 antibody can be identified and isolated by screening a recombinant combinatorial immunoglobulin library (*e.g.*, an antibody phage display library) with NIP45 to thereby isolate immunoglobulin library members that bind NIP45. Kits for generating and screening phage display libraries are commercially available (*e.g.*, the Pharmacia *Recombinant Phage Antibody System*, Catalog No. 27-9400-01; and the Stratagene *SurfZAP*™ *Phage Display Kit*, Catalog No. 240612). Additionally, examples of methods and reagents particularly amenable for use in generating and screening antibody display library can be found in, for example, Ladner *et al.* U.S. Patent No. 5,223,409; Kang *et al.* International Publication No. WO 92/18619; Dower *et al.* International Publication No. WO 91/17271; Winter *et al.* International Publication WO 92/20791; Markland *et al.* International Publication No. WO 92/15679; Breitling *et al.* International Publication WO 93/01288; McCafferty *et al.* International Publication No. WO 92/01047; Garrard *et al.* International Publication No. WO 92/09690; Ladner *et al.* International Publication No. WO 90/02809; Fuchs *et al.* (1991) *Bio/Technology* 9:1370-1372; Hay *et al.* (1992) *Hum Antibod Hybridomas* 3:81-85; Huse *et al.* (1989) *Science* 246:1275-1281; Griffiths *et al.* (1993) *EMBO J* 12:725-734; Hawkins *et al.* (1992) *J Mol Biol* 226:889-896; Clarkson *et al.* (1991) *Nature* 352:624-628; Gram *et al.* (1992) *PNAS* 89:3576-3580; Garrad *et al.* (1991) *Bio/Technology* 9:1373-1377; Hoogenboom *et al.* (1991) *Nuc Acid Res* 19:4133-4137;

Barbas *et al.* (1991) *PNAS* 88:7978-7982; and McCafferty *et al. Nature* (1990) 348:552-554.

Additionally, recombinant anti-NIP45 antibodies, such as chimeric and humanized monoclonal antibodies, comprising both human and non-human portions, which can be made using standard recombinant DNA techniques, are within the scope of the invention. Such chimeric and humanized monoclonal antibodies can be produced by recombinant DNA techniques known in the art, for example using methods described in Robinson *et al.* International Patent Publication PCT/US86/02269; Akira, *et al.* European Patent Application 184,187; Taniguchi, M., European Patent Application 171,496; Morrison *et al.* European Patent Application 173,494; Neuberger *et al.* PCT Application WO 86/01533; Cabilly *et al.* U.S. Patent No. 4,816,567; Cabilly *et al.* European Patent Application 125,023; Better *et al.* (1988) *Science* 240:1041-1043; Liu *et al.* (1987) *PNAS* 84:3439-3443; Liu *et al.* (1987) *J. Immunol.* 139:3521-3526; Sun *et al.* (1987) *PNAS* 84:214-218; Nishimura *et al.* (1987) *Canc. Res.* 47:999-1005; Wood *et al.* (1985) *Nature* 314:446-449; and Shaw *et al.* (1988) *J. Natl Cancer Inst.* 80:1553-1559); Morrison, S. L. (1985) *Science* 229:1202-1207; Oi *et al.* (1986) *BioTechniques* 4:214; Winter U.S. Patent 5,225,539; Jones *et al.* (1986) *Nature* 321:552-525; Verhoeyan *et al.* (1988) *Science* 239:1534; and Beidler *et al.* (1988) *J. Immunol.* 141:4053-4060.

An anti-NIP45 antibody (*e.g.*, monoclonal antibody) can be used to isolate NIP45 by standard techniques, such as affinity chromatography or immunoprecipitation. An anti-NIP45 antibody can facilitate the purification of natural NIP45 from cells and of recombinantly produced NIP45 expressed in host cells. Moreover, an anti-NIP45 antibody can be used to detect NIP45 protein (*e.g.*, in a cellular lysate or cell supernatant). Detection may be facilitated by coupling (*i.e.*, physically linking) the antibody to a detectable substance. Accordingly, in one embodiment, an anti-NIP45 antibody of the invention is labeled with a detectable substance. Examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, β-galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; and examples of suitable radioactive material include ^{125}I, ^{131}I, ^{35}S or ^{3}H.

D. Pharmaceutical Compositions

The NIP45 proteins and anti-NIP45 antibodies of the invention can be incorporated into pharmaceutical compositions suitable for administration. Such compositions typically comprise the protein or antibody and a pharmaceutically acceptable carrier. As used herein the term "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. For example, solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL™ (BASF, Parsippany, NJ) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyetheylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the

maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating the active compound (*e.g.*, a NIP45 protein or anti-NIP45 antibody) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Oral compositions generally include an inert diluent or an edible carrier. They can be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches, or capsules. Oral compositions can also be prepared using a fluid carrier for use as a mouthwash, wherein the compound in the fluid carrier is applied orally and swished and expectorated or swallowed. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

In one embodiment, the active compounds are prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate,

polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These may be prepared according to methods known to those skilled in the art, for example, as described in U.S. Patent No. 4,522,811.

E. Methods of Detecting NIP45 Activity

Another aspect of the invention pertains to a method of using the various NIP45 compositions of the invention. For example, the invention provides a method for detecting the presence of NIP45 protein or mRNA in a biological sample. The method involves contacting the biological sample with an agent capable of detecting NIP45 protein or mRNA such that the presence of NIP45 protein or mRNA is detected in the biological sample. A preferred agent for detecting NIP45 mRNA is a labeled nucleic acid probe capable of hybridizing to NIP45 mRNA. The nucleic acid probe can be, for example, the NIP45 cDNA of SEQ ID NO: 5, or a portion thereof, such as an oligonucleotide of at least 15, 30, 50, 100, 200, 300, 400, 500, 600, 700, 800, 900 or 1000 nucleotides in length and sufficient to specifically hybridize under stringent conditions to NIP45 mRNA. A preferred agent for detecting NIP45 protein is a labeled antibody capable of binding to NIP45 protein. Antibodies can be polyclonal, or more preferably, monoclonal. An intact antibody, or a fragment thereof (*e.g.*, Fab or $F(ab')_2$) can be used. The term "labeled", with regard to the probe or antibody, is intended to encompass direct labeling of the probe or antibody by coupling (*i.e.*, physically linking) a detectable substance to the probe or antibody, as well as indirect labeling of the probe or antibody by reactivity with another reagent that is directly labeled. Examples of indirect labeling include detection of a primary antibody using a fluorescently labeled secondary antibody and end-labeling of a DNA probe with biotin such that it can be detected with fluorescently labeled streptavidin. The term "biological sample" is intended to include tissues, cells and biological fluids. For example, techniques for detection of NIP45 mRNA include Northern hybridizations and *in situ* hybridizations. Techniques for detection of NIP45 protein include enzyme linked immunosorbent assays (ELISAs), Western blots, immunoprecipitations and immunofluorescence.

VI.. Combination Compositions and Kits

Compositions comprising combinations of modulatory agents are also provided by the invention. For example, two or more nucleotide sequences encoding transcription factors that regulate Th2-associated cytokine gene expression can be incorporated into a recombinant expression vector and introduced into a host cell. For example, the invention provides recombinant vectors, and host cells into which such vectors have been introduced, comprising a first nucleotide sequence encoding a first transcription factor that cooperates with an NF-AT family protein to regulate expression of the Th2-associated cytokine gene and a second nucleotide sequence encoding a second transcription factor that contributes to the regulation of the Th2-associated cytokine gene. Preferably, the first nucleotide sequence encodes a maf family protein (*e.g.*, c-Maf) or an NF-AT-interacting protein (*e.g.*, NIP45). Preferably, the second nucleotide sequence encodes a transcription factor selected from the group consisting of NF-AT family proteins, NF-AT-interacting proteins, maf family proteins and AP-1 family proteins.

Kits for modulating Th2-associated cytokine production or Th1/Th2 subset development are also encompassed by the invention. In one embodiment, a kit of the invention comprises at least one modulatory agent of the invention packaged with instructions for using the modulatory agent to modulate Th2-associated cytokine production or Th1/Th2 subset development. In one embodiment, the kit comprises at least one stimulatory agent for use in stimulating Th2-associated cytokine production or upregulating Th2 subset development (or downregulating Th1 subset development). In another embodiment, the kit comprises at least one inhibitory agent for use in inhibiting Th2-associated cytokine production or downregulating Th2 subset development (or upregulating Th1 subset development). Combination kits, comprising two or more of the modulatory (*e.g.*, stimulatory or inhibitory) agents of the invention are also provided.

VII. Screening Assays

Another aspect of the invention pertains to screening assays for identifying compounds that modulate the activity of a transcription factor that regulates expression of a Th2-associated cytokine gene. In various embodiments, these screening assays can identify, for example, compounds that modulate the expression or functional activity of the transcription factor, proteins that interact with the transcription factor, as well as compounds that modulate these protein-protein interactions, and compounds that modulate the interaction of the transcription factor with a *cis*-acting target site (*e.g.*, a MARE) within a Th2-associated cytokine gene.

In one embodiment, the invention provides a method for identifying a compound that modulates the activity of a transcription factor that cooperates with a Nuclear Factor of Activated T cells (NF-AT) family protein to regulate expression of a Th2-associated cytokine gene, comprising

providing a indicator composition having activity of a transcription factor that cooperates with an NFAT family protein to regulate expression of the Th2-associated cytokine gene;

contacting the indicator composition with a test compound; and

determining the effect of the test compound on the activity of the transcription factor in the indicator composition to thereby identify a compound that modulates the activity of a transcription factor that cooperates with an NFAT family protein to regulate expression of a Th2-associated cytokine gene.

The transcription factor can be, for example, a maf family protein, such as c-Maf or a small maf protein (e.g., p18). Alternatively, the transcription factor can be a factor interacts with an NF-AT family protein, such as NIP45.

The indicator composition can be a cell-free composition or it can be a cellular composition. For example, in one embodiment, the indicator composition is a lymphoid cell, such as a Th2 cell. In another embodiment, the indicator composition is a yeast cell.

In a preferred embodiment, the indicator composition comprises an indicator cell, wherein said indicator cell comprises: (i) the transcription factor and (ii) a reporter gene responsive to the transcription factor. Preferably, the indicator cell contains:

i) a recombinant expression vector encoding the transcription factor; and

ii) a vector comprising regulatory sequences of a Th2-associated cytokine gene operatively linked a reporter gene; and

said method comprises:

a) contacting the indicator cell with a test compound;

b) determining the level of expression of the reporter gene in the indicator cell in the presence of the test compound; and

c) comparing the level of expression of the reporter gene in the indicator cell in the presence of the test compound with the level of expression of the reporter gene in the indicator cell in the absence of the test compound to thereby identify a compound that modulates the activity of the transcription factor.

In another preferred embodiment, the indicator composition comprises a preparation of: (i) the transcription factor and (ii) a DNA molecule to which the transcription factor binds, and

said method comprises:

a) contacting the indicator composition with a test compound;

b) determining the degree of interaction of the transcription factor and the DNA molecule in the presence of the test compound; and

c) comparing the degree of interaction of the transcription factor and the DNA molecule in the presence of the test compound with the degree of interaction of the transcription factor and the DNA molecule in the absence of the test compound to thereby identify a compound that modulates the activity of the transcription factor.

In one embodiment of the foregoing method, the transcription factor is a maf family protein and the DNA molecule comprises a maf response element (MARE).

In another preferred embodiment, the method identifies a protein from Th2 cells that interacts with the transcription factor. In this embodiment,

the indicator composition is an indicator cell, which indicator cell comprises:

i) a reporter gene operably linked to a transcriptional regulatory sequence; and

ii) a first chimeric gene which encodes a first fusion protein, said first fusion protein including the transcription factor that cooperates with an NFAT family protein to regulate expression of a Th2-associated cytokine gene;

the test compound comprises a library of second chimeric genes, which library encodes second fusion proteins, the second fusion proteins including proteins derived from Th2 cells;

expression of the reporter gene being sensitive to interactions between the first fusion protein, the second fusion protein and the transcriptional regulatory sequence; and

wherein the effect of the test compound on the transcription factor in the indicator composition is determined by determining the level of expression of the reporter gene in the indicator cell to thereby identify a test compound comprising a protein from Th2 cells that interacts with the transcription factor.

The invention further provides a method for identifying a compound that modulates an interaction between NIP45 and an NF-AT family protein, comprising:

a) combining:

(i) NIP45, or an NF-AT-interacting portion thereof; and

(ii) an NF-AT family protein, or a NIP45-interacting portion thereof;

in the presence and absence of a test compound;

b) determining the degree of interaction between (i) and (ii) in the presence and absence of the test compound; and

c) identifying a compound that modulates an interaction between NIP45 and an NF-AT family protein.

Preferably, the NIP45-interacting portion of the NF-AT family protein comprises the Rel Homology Domain of the NF-AT family protein. In one embodiment, the degree of interaction between (i) and (ii) is determined by labeling (i) or (ii) with a detectable substance, isolating non-labeled (i) or (ii) and quantitating the amount of labeled (i) or (ii) that has become associated with non-labeled (i) or (ii). The method can be used to identify compounds that either increase or decrease the interaction between NIP45 and an NF-AT family protein.

In a preferred embodiment of the screening assays of the invention, once a test compound is identified as modulating the activity of a transcription factor of interest, the effect of the test compound on an immune response is then tested. Accordingly, the screening methods of the invention can further comprise determining the effect of the compound on an immune response to thereby identify a compound that modulates an immune response. In one embodiment, the effect of the compound on an immune response is determined by determining the effect of the compound on expression of a Th2-associated cytokine gene, such as an interleukin-4 gene. In another embodiment, the effect of the compound of interest on an immune response is determined by determining the effect of the compound on development of T helper type 1 (Th1) or T helper type 2 (Th2) cells.

Recombinant expression vectors that can be used for expression of a transcription factor in the indicator cell are known in the art (see discussions above and also the Examples). In one embodiment, within the expression vector the transcription factor-coding sequences are operatively linked to regulatory sequences that allow for constitutive expression of the transcription factor in the indicator cell (*e.g.*, viral regulatory sequences, such as a cytomegalovirus promoter/enhancer, can be used). Use of a recombinant expression vector that allows for constitutive expression of the transcription factor in the indicator cell is preferred for identification of compounds that enhance or inhibit the activity of the transcription factor. In an alternative embodiment,

within the expression vector the transcription factor-coding sequences are operatively linked to regulatory sequences of the endogenous corresponding transcription factor gene (*i.e.*, the promoter regulatory region derived from the endogenous gene). Use of a recombinant expression vector in which transcription factor expression is controlled by the endogenous regulatory sequences is preferred for identification of compounds that enhance or inhibit the transcriptional expression of the transcription factor.

In methods in which a Th2-associated cytokine gene is utilized, preferably, the Th2-associated cytokine is interleukin-4. It has previously shown that Th2-specific, inducible IL-4 expression can be directed by as little as 157 bp of the proximal IL-4 promoter in Th2 cells (Hodge, M. *et al.* (1995) *J. Immunol.* 154:6397-6405). Accordingly, in one embodiment, a method of the invention utilizes a reporter gene construct containing this region of the proximal IL-4 promoter, most preferably nucleotides -157 to +58 (relative to the start site of transcription at +1) of the IL-4 promoter. Alternatively, stronger reporter gene expression can be achieved using a longer portion of the IL-4 upstream regulatory region, such as about 3 kb of upstream regulatory sequences. Suitable reporter gene constructs are described in Todd, M. *et al.* (1993) *J. Exp. Med.* 177:1663-1674. See also the Examples for descriptions of IL-4 reporter gene constructs.

A variety of reporter genes are known in the art and are suitable for use in the screening assays of the invention. Examples of suitable reporter genes include those which encode chloramphenicol acetyltransferase, beta-galactosidase, alkaline phosphatase or luciferase. Standard methods for measuring the activity of these gene products are known in the art.

A variety of cell types are suitable for use as an indicator cell in the screening assay. Preferably a cell line is used which does not normally express c-Maf, such as a B cell (*e.g.*, the M12 B lymphoma cell line) or a Th1 cell clone (*e.g.*, AE7 cells). Nonlymphoid cell lines can also be used as indicator cells, such as the HepG2 hepatoma cell line.

In one embodiment, the level of expression of the reporter gene in the indicator cell in the presence of the test compound is higher than the level of expression of the reporter gene in the indicator cell in the absence of the test compound and the test compound is identified as a compound that stimulates the expression or activity of the transcription factor. In another embodiment, the level of expression of the reporter gene in the indicator cell in the presence of the test compound is lower than the level of expression of the reporter gene in the indicator cell in the absence of the test compound

and the test compound is identified as a compound that inhibits the expression or activity of the transcription factor.

Alternative to the use of a reporter gene construct, compounds that modulate the expression or activity of a transcription factor can be identified by using other "read-outs." For example, an indicator cell can be transfected with a transcription factor expression vector, incubated in the presence and in the absence of a test compound, and Th2-associated cytokine production can be assessed by detecting cytokine mRNA (*e.g.*, IL-4 mRNA) in the indicator cell or cytokine secretion (*i.e.*, IL-4 secretion) into the culture supernatant. Standard methods for detecting cytokine mRNA, such as reverse transcription-polymerase chain reaction (RT-PCR) are known in the art. Standard methods for detecting cytokine protein in culture supernatants, such as enzyme linked immunosorbent assays (ELISA) are also known in the art. For further descriptions of methods for detecting cytokine mRNA and/or protein, see also the Examples.

As described above, the invention provides a screening assay for identifying proteins (*e.g.*, proteins in Th2 cells) that interact with a transcription factor of interest, *e.g.*, c-Maf or NF-AT or NIP45. These assays can be designed based on the two-hybrid assay system (also referred to as an interaction trap assay) known in the art (see *e.g.*, Field U.S. Patent No. 5,283,173; Zervos *et al.* (1993) *Cell* 72:223-232; Madura *et al.* (1993) *J. Biol. Chem.* 268:12046-12054; Bartel *et al.* (1993) *Biotechniques* 14:920-924; and Iwabuchi *et al.* (1993) *Oncogene* 8:1693-1696). The two-hybrid assay is generally used for identifying proteins that interact with a particular target protein. The assay employs gene fusions to identify proteins capable of interacting to reconstitute a functional transcriptional activator. The transcriptional activator consists of a DNA-binding domain and a transcriptional activation domain, wherein both domains are required to activate transcription of genes downstream from a target sequence (such as an upstream activator sequence (UAS) for GAL4). DNA sequences encoding a target "bait" protein are fused to either of these domains and a library of DNA sequences is fused to the other domain. "Fish" fusion proteins (generated from the fusion library) capable of binding to the target-fusion protein (*e.g.*, a target GAL4-fusion "bait') will generally bring the two domains (DNA-binding domain and transcriptional activation domain) into close enough proximity to activate the transcription of a reporter gene inserted downstream from the target sequence. Thus, the "fish" proteins can be identified by their ability to reconstitute a functional transcriptional activator (*e.g.*, a functional GAL4 transactivator).

This general two-hybrid system can be applied to the identification of proteins in Th2 cells that interact with c-Maf (or, using similar methods, with other transcription factors of interest) by construction of a target c-Maf fusion protein (*e.g.*, a c-Maf/GAL4 binding domain fusion as the "bait") and a cDNA library of "fish" fusion proteins (*e.g.*, a cDNA/GAL4 activation domain library), wherein the cDNA library is prepared from mRNA of Th2 cells, and introducing these constructs into a host cell that also contains a reporter gene construct linked to a regulatory sequence responsive to c-Maf (*e.g.*, a MARE sequence, for example a region of the IL-4 promoter, as discussed above). cDNAs encoding proteins from Th2 cells that interact with c-Maf can be identified based upon transactivation of the reporter gene construct.

Alternatively, a "single-hybrid" assay, such as that described in Sieweke, M.H. *et al.* (1996) *Cell* 85:49-60, can be used to identify proteins from Th2 cells that interact with c-Maf. This assay is a modification of the two-hybrid system discussed above. In this system, the "bait" is a transcription factor from which the transactivation domain has been removed (*e.g.*, c-Maf from which the amino-terminal transactivation domain has been removed) and the "fish" is a non-fusion cDNA library (*e.g.*, a cDNA library prepared from Th2 cells). These constructs are introduced into host cells (*e.g.*, yeast cells) that also contains a reporter gene construct linked to a regulatory sequence responsive to the transcription factor (*e.g.*, a MARE sequence, for example a region of the IL-4 promoter, responsive to c-Maf). cDNAs encoding proteins from Th2 cells that interact with c-Maf (or other transcription factor of interest) can be identified based upon transactivation of the reporter gene construct.

As described above, the invention provides a screening assay for identifying compounds that modulate the interaction of a transcription factor of the invention with a DNA molecule to which the transcription factor binds, such as c-Maf and a MARE in an IL-4 gene regulatory region, respectively. Assays are known in the art that detect the interaction of a DNA binding protein with a target DNA sequence (*e.g.*, electrophoretic mobility shift assays, DNAse I footprinting assays and the like; for further descriptions see the Examples). By performing such assays in the presence and absence of test compounds, these assays can be used to identify compounds that modulate (*e.g.*, inhibit or enhance) the interaction of the DNA binding protein with its target DNA sequence.

In one embodiment, the amount of binding of the transcription factor to the DNA fragment in the presence of the test compound is greater than the amount of binding of the transcription factor to the DNA fragment in the absence of the test compound, in which case the test compound is identified as a compound that enhances binding of the

transcription factor. In another embodiment, the amount of binding of the transcription factor to the DNA fragment in the presence of the test compound is less than the amount of binding of the transcription factor to the DNA fragment in the absence of the test compound, in which case the test compound is identified as a compound that inhibits binding of the transcription factor.

In the methods of the invention for identifying agents that modulate an interaction between NIP45 and an NF-AT family protein, isolated NIP45 and/or NF-AT family proteins may be used in the method, or, alternatively, only portions of NIP45 and/or an NF-AT family protein may be used. For example, an isolated NF-AT Rel Homology Domain (or a larger subregion of NF-AT that includes the RHD) can be used as the NIP45-interacting portion of NF-AT. Likewise, a portion of NIP45 capable of binding to the NF-AT RHD may be used. In a preferred embodiment, one or both of (i) and (ii) are fusion proteins, such as GST fusion proteins (*e.g.*, GST-NF-AT RHD can be used as the NIP45-interacting portion of NF-AT). The degree of interaction between (i) and (ii) can be determined, for example, by labeling one of the proteins with a detectable substance (*e.g.*, a radiolabel), isolating the non-labeled protein and quantitating the amount of detectable substance that has become associated with the non-labeled protein. The assay can be used to identify agents that either stimulate or inhibit the interaction between NIP45 and an NF-AT family protein. An agent that stimulates the interaction between NIP45 and an NF-AT family protein is identified based upon its ability to increase the degree of interaction between (i) and (ii) as compared to the degree of interaction in the absence of the agent, whereas an agent that inhibits the interaction between NIP45 and an NF-AT family protein is identified based upon its ability to decrease the degree of interaction between (i) and (ii) as compared to the degree of interaction in the absence of the agent. Assays systems for identifying agents that modulate SH2 domain-ligand interactions as described in U.S. Patent No. 5,352,660 by Pawson can be adapted to identifying agents that modulate the NIP45/NF-AT RHD interaction.

This invention is further illustrated by the following examples which should not be construed as limiting. The contents of all references, patents and published patent applications cited throughout this application are hereby incorporated by reference. Nucleotide and amino acid sequences deposited in public databases as referred to herein are also hereby incorporated by reference.

EXAMPLE 1: **Cytokine Specificity is Due to a Positive Transacting Factor and Not to a Repressor**

Tissue specificity can be achieved through the action of repressor or silencer proteins. Thus it was possible that the IL-2 and IL-4 genes were actively repressed in Th2 and Th1 cells respectively. To test for the existence of repressor proteins, somatic cell fusions were performed between a Th1(D1.1) and a Th2 (D10) clone of differing MHC Class I haplotypes. The Th1 clone D1.1 (K^d) and the Th2 clone D10 (K^k) were fused according to the "suspension cell fusion" procedure (Lane, R.D. *et al.* (1986) *Methods Enzymol.* 121:183-192). After fusion, the cells were allowed to recover for 8 hours and then double- stained using PE-conjugated anti-K^k and FITC-conjugated anti-K^d antibodies (Pharmingen, La Jolla, CA). Cells were then sorted on the basis of size to distinguish unfused cells from hetero and homokaryons and by fluorescence to identify single-positive and double-positive cells. As indicated in the schematic of this approach shown in Figure 1A, three populations were sorted for: large PE-positive cells (D1.1 x D1.1), large FITC-positive cells (D10 x D10), and large PE and FITC positive cells (D1.1 x D10). Cells expressing both MHC class I K^b and K^k markers were heterokaryons while cells expressing only K^b or K^k represented homokaryons and served as controls.

The three populations were then stimulated in culture with antibodies to CD3 to activate cytokine gene expression and RNA prepared for RT-PCR and Northern blot analysis. Approximately 5×10^5 cells were obtained for each population. Routinely, 5-10% of the cells had undergone fusion. Each of these three populations was then split in half, one half transferred to pre-rinsed anti-CD3 coated plates, the remaining half to uncoated plates. After four hours, the cells were harvested, and poly(A+) RNA isolated using the Micro-FastTrackTM kit (Stratagene, La Jolla, CA). cDNA was made using the SuperScript kit (Gibco/BRL, Bethesda, MD), and used for PCR analysis using commercially available primers specific for murine IL-2, IL-4 and β-actin according to the manufacturer's instructions (Stratagene, La Jolla, CA). PCR reactions included 0.5 μ Ci α-^{32}P-dCTP (3000 Ci/mmol, NEN Dupont). PCR products were ethanol precipitated, separated by nondenaturing PAGE and dried and visualized by autoradiography.

The results of the RT-PCT analysis of cytokine mRNA expression are shown in Figure 1B. The Th1 and Th2 clones and the Th homokaryons transcribed only IL-2 (Th1) or IL-4 (Th2) respectively, while the Th1/Th2 heterokaryons produced both cytokines. In contrast, the existence of repressor protein(s) should have resulted in the

extinction of both cytokines in the heterokaryons. From these experiments, it was concluded that cytokine specificity in Th1 vs. Th2 cells was mediated by Th-specific positive transacting factors rather than by selective silencer proteins.

EXAMPLE 2: **Isolation of a Th2-Specific *c-maf* Gene from a cDNA Library Prepared from an Anti-CD3 Activated Th2 Clone**

In the course of screening a cDNA library prepared from an anti-CD3 activated Th2 clone, D10, for NF-AT-interacting proteins by the yeast two-hybrid system (for descriptions of this system, see *e.g.*, Field U.S. Patent No. 5,283,173; Zervos *et al.* (1993) *Cell* 72:223-232; Madura *et al.* (1993) *J. Biol. Chem.* 268:12046-12054; Bartel *et al.* (1993) *Biotechniques* 14:920-924; and Iwabuchi *et al.* (1993) *Oncogene* 8:1693-1696), multiple cDNAs were isolated, all of which were extremely weak interactors. All cDNAs obtained in this screen were next evaluated for Th-specific expression by Northern blot analysis using a panel of Th1 and Th2 clones. One such cDNA, which was repeatedly isolated (60 of 140) detected transcripts only in RNA prepared from Th2 clones (D10, CDC35) and not from either Th1 clones (AR5, OS6, D1) or from a B cell lymphoma, M12, as illustrated in the Northern blot analysis depicted in Figure 2A. Further, the levels of transcripts detected in D10 Th2 cells were substantially increased upon activation by ligation of the T cell receptor with anti-CD3 antibody. No induction of the transcript detected by this cDNA clone occurred in Th1 clones upon anti-CD3 treatment. A control probe, GAPDH, demonstrated approximately equal loading of RNA in all lanes. Thus, the expression of this cDNA clone in the lymphoid lineage appeared to be Th2-specific and sensitive to signals transmitted through the T cell receptor. For these Northern blots, total RNA was prepared by using Trizol (GIBCO/BRL) according to manufacturer's instructions. 10 μg of total RNA from each sample was fractionated on a formaldehyde agarose gel and transferred to a nylon membrane. A 300 bp DraI fragment derived from the 3' untranslated region of the isolated clone was labeled with α-^{32}P-dCTP using Random Primed DNA Labeling Kit (Boehringer Mannheim, Indianapolis, IN). Hybridization was performed using QuikHyb (Stratagene, La Jolla, CA) according to manufacturer's instructions.

To determine whether the expression of this gene was tissue-specific and regulated during the course of normal Th cell development, the following experiment was performed. Naive spleen cells (Th precursor (Thp) cells) were driven along a Th1 or Th2 pathway by treatment with anti-CD3 in the presence of cytokines and anti-cytokine antibodies (IFNγ and anti-IL-4 for Th1, IL-4 and anti-IFNg for Th2). Splenic

cell suspensions were prepared from 6-8 week-old Balb/c mice, cultured in RPMI 1640 supplemented with 10% FCS at a density of 10^6 cells/ml, and stimulated with plate bound anti-CD3 antibody in the presence of 5 μg/ml of anti-IL4 antibody (11B11) for the Th1 lineage, or 5 μg/ml of anti-IFNγ antibody (XMG-1) for the Th2 lineage. 24 hours after stimulation, 50 U/ml IL2 was added to all cultures, and 500 U/ml IL4 (Genzyme) was added to Th2 cultures. 7 days after the primary stimulation, all cells were harvested, washed and restimulated with plate bound anti-CD3 antibody. Northern blot analysis of differentiating cells harvested at various time points after stimulation in a primary (day 0-8) and secondary (0-20 hours) response was performed, using the methodology described above, and identification of differentiating Thp cells as Th1 or Th2 was determined by analyzing culture supernatants by ELISA for IL-10 and IFNγ. ELISA for cytokine quantitation was performed as follows. All anti-cytokine antibodies were purchased from Pharmingen. ELISA was performed according to Pharmingen's instructions with the exception that Avidin-Alkaline Phosphatase (Sigma) at 1:500 dilution in PBS/BSA was used in place of avidin-peroxidase. P-nitrophenyl phosphate (GIBCO BRL) at 4 mg/ml in substrate buffer (10% diethanolamine, 0.5 mM $MgCl_2$, 0.02% sodium azide, pH 9.8) was used as substrate.

In two independent experiments, representative results of which are shown in Figure 2B, this analysis revealed low level or undetectable expression of this cDNA in naive spleen cells at baseline at day 0. In cultures differentiating along a Th2 pathway, substantial induction of transcripts occurred by day 8 in a primary stimulation and by 20 hr in a secondary stimulation. In contrast, no induction occurred in cells being driven along a Th1 pathway. A control probe (GAPDH) showed approximately equal loading of RNA in all lanes. The low level of transcripts present in cells being driven along a Th1 pathway likely reflects the presence of residual Th2 cells since complete skewing does not occur in this *in vitro* differentiation system.

Together, these experiments revealed that the isolated cDNA is selectively expressed in Th2 clones, where it is induced upon T cell activation, and that it is absent from Th1 clones and a B lymphoma. Further, this gene is induced in normal Thp when they are driven towards the Th2 lineage, but is not induced during Th1 development.

The cDNA obtained from the yeast two-hybrid screen was used as a probe to isolate a full-length cDNA from a D10 Th2 cell cDNA library by standard hybridization methods. A 4.3 kb cDNA clone was isolated from the Th2 cell library and sequenced by standard methods. Sequence analysis revealed that this Th2-specific gene corresponded in sequence to the *c-maf* proto-oncogene.

EXAMPLE 3: Ectopic Expression of c-Maf in Th1 and B Cells Results in Activation of the IL-4 Promoter

The identification of the isolated cDNA described in Example 2 as a member of the AP-1/CREB/ATF gene family, together with its selective expression in Th2 cells raised the possibility that c-Maf controlled the tissue-specific transcription of the IL-4 gene. Additionally, the presence of transcripts encoding c-maf correlated well with IL-4 expression in Th2 cells and in three of four transformed mast cell lines examined. To test whether c-Maf could transactivate the IL-4 promoter, cotransfection experiments were performed.

Th1 clones and the B lymphoma M12.4.C3 (M12) neither express *c-maf* nor transcribe the IL-4 gene. If c-Maf is the transcription factor critical for controlling IL-4 gene expression, then forced expression in these cells should permit IL-4 gene expression. To test this, the full-length (4.3 kb) c-maf cDNA clone was inserted into the SalI site of the
pMex-NeoI mammalian expression vector, which utilizes the CMV enhancer to drive expression of the inserted sequence. The c-Maf expression vector was then cotransfected with an IL-4 promoter reporter construct into the Th1 clone AE7 and the B lymphoma M12. The generation of the wild type IL4 CAT reporter construct, containing an IL4 promoter fragment from -157 to +68 operatively linked to a chloramphenicol acetyltransferase gene is described in Hodge, M. *et al.* (1995) *J. Immunol.* 154:6397-6405. The Th1 clone was cultured in RPMI 1640 supplemented with 10% FCS and 10% Con-A stimulated rat splenocyte supernatant, and maintained by bi-weekly stimulation with appropriate antigen and APCs. M12 cells were cultured in RPMI 1640 supplemented with 10% FCS.

The Th1 clone AE7 or M12 B lymphoma cells were transiently transfected by preincubating 0.4 ml of cells, containing 2×10^7 cells/ml AE7 or 3×10^6 cells/ml M12 cells in serum-free RPMI 1640 with 20 μg (AE7) or 5 μg (M12) of each plasmid for 10 minutes at room temperature. The samples were then electroporated using a BIO-RAD Gene Pulser (BIO-RAD, Richmond, CA) set at 975 μF, 280 V, and immediately placed on ice for 10 minutes. The transfected cells were allowed to recover overnight in complete media and stimulated with plate bound anti-CD3 antibody ({Pharmingen, San Diego, CA} 1 μg/ml in 1XPBS overnight at 4 °C) or with 50 ng/ml PMA (Sigma, St. Louis, MO) and 1 μM Ionomycin (Calbiochem Corp., La Jolla, California). for 24 hours. Cell lysate was prepared by freeze-thaw lysis in 0.25 M Tris-Cl, pH 7.8. Equal amounts

of protein (between 5-20 μg) were used for CAT assays. CAT assays were performed as described in Todd, M. *et al.* (1993) *J. Exp. Med.* 177:1663-1674.

It has previously shown that Th2-specific, inducible IL-4 expression can be directed by as little as 157 bp of the proximal IL-4 promoter in Th2 cells (Hodge, M. *et al.* (1995) *J. Immunol.* 154:6397-6405). In cotransfection experiments, the results of which are summarized in Figure 3A, it is demonstrated that ectopic expression of c-Maf in the Th1 clone AE7 results in substantial activity of the IL-4 promoter reporter after stimulation through the T cell receptor. The fold induction observed was approximately 5 fold over that observed with the control empty vector alone. Although expression of a reporter construct containing proximal (-157 to +58) IL-4 promoter sequences in the subclone of AE7 cells utilized here has not been previously observed, it has been demonstrated that small amounts of IL-4 mRNA can be detected by RT-PCR in other subclones of AE7. To more rigorously test the ability of c-Maf to transactivate the IL-4 promoter in a non-IL-4 producing cell, the same experiment was performed in the B lymphoma cell line, M12. Normal B cells and B lymphoma cells do not produce IL-4. Representative results of the cotransfection experiments are depicted in Figure 3B and a summary of three independent experiments is shown below in Table 1.

Table 1

	CAT Activity (fold induction)			
Plasmids	**PMA/iono.**	**Exp. I***	**Exp. II**	**Exp. III**
pMEX-NeoI/pREP4	-	1	1	1
	+	7.6	1	1.4
pMEX-Maf/pREP4	-	95	5	18.6
	+	186	7	37
pMEX-c-Fos/pREP4	-	2.7	1	0.8
	+	7.6	1.2	1
pMEX-JunD/pREP4	-	ND**	0.9	0.5
	+	ND	1.4	1.9
pMEX-NeoI/pREP4-NF-ATp	-	14.2	1.6	0.3
	+	41.2	3.5	0.3
pMEX-Maf/pREP4-NF-ATp	-	136	54	26.3
	+	138	100	54.7
pMEX-c-Fos/pREP4-NF-ATp	-	7.4	1.6	3
	+	15.4	1.9	6.1

* In experiment I, 20 mg of cell lysate was incubated for 2 hours. In experiments II and III, only 5 mg of cell lysate was incubated for 1 hour in order to reveal synergy between c-Maf and NF-ATp

**ND = not done

The results in M12 B lymphoma cells confirmed the findings in the Th1 clone. Ectopic expression of c-Maf resulted in substantial activity of the IL-4 promoter in M12 cells, either unstimulated or stimulated with PMA/Ca++ ionophore. The fold induction observed when compared to transfection of a control vector averaged approximately 50 in unstimulated M12 cells. Stimulation of M12 cells with PMA/Ca++ ionophore, which should result in translocation of NF-ATs to the nucleus and induction of other AP-1 family members (Flanagan, W.M. (1991) *Nature* 352:803-807; Jain, J. *et al.* (1993) *Nature* 365:352-355), increased the basal activity of the IL-4 promoter, but a marked induction in promoter activity by c-Maf was still present (average of approximately 25 fold). C-Maf did not transactivate a control reporter driven by NF-AT multimers, demonstrating the specificity of transactivation.

As a control for the specificity of c-Maf as opposed to other AP-1 family members, the c-Fos and c-Jun proteins were also overexpressed in M12 cells utilizing murine full-length cDNAs encoding c-Fos and JunD in the mammalian expression vector of pMEX-NeoI together with the IL-4 reporter plasmid. No IL-4 promoter activity could be achieved by overexpression of either of these two AP-1 family members in M12 cells. Thus, c-Maf has a unique ability to drive IL-4 gene transcription in M12 B cells. Further, forced expression of c-Maf in the hepatoma cell line HepG2 also resulted in IL-4 promoter transactivation. These experiments demonstrate that the provision of c-Maf to c-Maf negative Th1 or B cells, or to non-lymphoid cells (*e.g.*, a hepatoma cell line) , permits the cells to transactivate the IL-4 promoter.

NF-AT proteins have been shown to be critically important in the regulation of both the IL-4 and IL-2 cytokines. NF-ATp was the first member of this family to be isolated (McCaffrey, P.G. *et al.* (1993) *Science* 262:750-754). Both AE7 and M12 cells have endogenous NF-ATp protein, but nevertheless do not transcribe IL-4. Although NF-ATp could not therefore account for selective IL-4 gene transcription, it was of interest to test whether overexpression of NF-ATp in unstimulated or stimulated M12 cells would further increase the transactivation of the IL-4 promoter by c-maf. M12 cells were cotransfected with the IL-4 reporter construct and either an NFAPp expression vector (pREP$_4$-NF-ATp, which also carries a hygromycin resistance gene) alone or the NFAPp expression vector together with the c-Maf expression vector. Overexpression of NF-ATp alone in M12 cells resulted in some modest transactivation of the IL-4 promoter. This transactivation was markedly increased by ectopic expression of c-Maf, an increase which was not just additive but was synergistic (see Fig. 3B and Table 1). In contrast, c-Fos overexpression did not further increase the

modest transactivation achieved by NF-ATp. These results indicate that c-maf and NF-ATp interact to achieve maximal induction of the IL-4 promoter, the tissue-specificity being provided by
c-Maf.

EXAMPLE 4: Ectopic Expression of C-Maf Activates Transcription of the Endogenous IL-4 Gene in a B Lymphoma

As demonstrated in Example 3, c-Maf transactivates the IL-4 promoter in transient transfection assays in Th1, B and non-lymphoid cells. To test whether expression of c-maf in non-IL-4 producing cells can activate the transcription of endogenous IL-4, the B lymphoma M12 was stably transfected with expression vectors encoding c-maf, NF-ATp or both, or junD with and without NF-ATp as a control. For stable transfection, M12 cells were transfected as described above in Example 3. The transfected cells were allowed to recover in complete media for 48 hours before the addition of Neomycin (GIBCO/BRL, Gaithersburg, MD) and Hygromycin (Calbiochem, Corp.) at a concentration of 400 μg/ml of each antibiotic. The transfected cells were supplemented with fresh media every other day.

Stably transfected M12 cells were plated at equal density supernatants harvested 24 hours later to measure cytokines by ELISA. ELISAs were performed as described in Example 2. The results, shown in Figure 4, demonstrate that in these experiments M12 cells transfected with c-maf, junD or NF-ATp alone did not produce measurable IL-4 by ELISA. However, M12 cells stably transfected with both c-maf and NF-ATp did produce detectable, but low level, IL-4 by ELISA. These results were confirmed by RT-PCR on RNA from these transfected cells. In contrast, these cells did not produce detectable IL-2. The requirement for both c-maf and NF-ATp is consistent with the synergistic effect of these factors in the transactivation of the IL-4 promoter noted in the transient transfection experiments in M12 cells. In contrast, transfection of junD, an AP-1 family member which can increase IL-4 expression in Th2 cells, alone or together with NF-ATp, did not result in IL-4 production. These results demonstrate the essential and selective role of c-Maf in directing tissue-specific endogenous IL-4 production.

EXAMPLE 5: A Site in the IL-4 Promoter is Footprinted by Extracts from Th2 but not Th1 Clones

The experiments described in Examples 3 and 4 demonstrated a clear functional role for c-maf in controlling tissue-specific expression of IL-4. Further, c-maf transcripts were expressed in Th2 but not Th1 cells. However, DNA-protein complexes were not detected by electrophoretic mobility shift assays (EMSA) when using nuclear extracts prepared from Th2 cells. To further examine whether a protein in Th2 nuclear extracts might bind to the MARE, or nearby sequences, the more sensitive technique of DNAseI footprinting was used. Two Th2 clones (D10, CDC35) and two Th1 clones (AE7, S53) were activated by ligation of the T cell receptor with plate-bound anti-CD3 antibody, and nuclear extracts prepared at time 0 (unstimulated), 2 hours and 6 hours later. DNAseI footprinting analysis was then performed according to standard methods using a Klenow end-labeled IL-4 promoter fragment (-157 to +68). The results are shown in Figure 5A. Stimulated extracts from both Th1 and Th2 cells footprinted the two NF-AT sites and the AP-1 site upstream of the distal NF-AT site as described previously (Rooney, J. *et al.* (1995) *Immunity* 2:545-553), consistent with the demonstrated function of NF-AT and AP-1 proteins in regulating both the IL-2 and the IL-4 promoters (Rooney, J. *et al.* (1995) *Immunity* 2:545-553; Rooney, J. *et al.* (1995) *Mol. Cell. Biol.* 15:6299-6310). Furthermore, inspection of the autoradiograph revealed an area of hypersensitivity on the non-coding strand at residues -28 and -29 when extracts from stimulated Th2 but not stimulated Th1 cells were used. Unstimulated Th cell extracts did not footprint this region. The Th2 footprint observed was subtle, but reproducible in two experiments and is located in a site that has previously been demonstrated to be critical for IL-4 promoter activation in Th2 cells (Hodge, M. *et al.* (1995) *J. Immunol.* 154:6397-6405). A schematic summary of sites occupied in the IL-4 promoter as detected by footprint analysis is shown in Figure 5B. These results indicate that a site in the proximal IL-4 promoter, previously shown to be functionally important, is occupied in activated Th2 but not in activated Th1 cells.

EXAMPLE 6: Recombinant c-maf Binds to a MARE Site in the IL-4 Promoter

The Th2-specific footprint does not contain a c-maf response element (MARE). However, examination of the proximal IL-4 promoter revealed a half c-maf binding site (MARE) (residues -42 to -37) immediately downstream of the proximal NF-AT site (residues -56 to -51) (shown schematically in Fig 5B). It has previously been

demonstrated that mutation of this site abolished activity of the IL-4 promoter in Th2 cells (Hodge, M. *et al.* (1995) *J. Immunol.* 154:6397-6405). To determine if c-Maf bound this site, a truncated

c-Maf recombinant protein containing the b-zip domain(amino acids 171-371) was expressed from *E. coli*, purified on an S-Tag agarose column and used in electrophoretic mobility shift assays with radiolabeled MARE oligonucleotide.

The expression vector for recombinant c-Maf was constructed by inserting a cDNA fragment encoding a.a. residues 171 to 371 of c-Maf (disclosed in Kurschner C. and Morgan, J.I. (1995) *Mol. Cell. Biol.* 15:246-254) into the NotI site of pET29 (Novagen, Inc. Madison, WI). The truncated c-Maf protein was expressed using T7 polymerase in the BL21(DE3)strain. Cells were induced by the addition of 1 mM IPTG and incubated at 37° C for 3 hours. The induced cells were lysed in 1 X Bind/Wash buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Triton X-100) followed by sonication. The c-Maf protein was then purified from the soluble fraction by using the S-Tag Purification Kit (Novagen) according to manufacturer's instructions. Two additional proteins, NF-ATp and c-Jun, were also used in EMSA assays. The recombinant NF-ATp, containing the Rel domain of murine NF-ATp, was expressed using an *in vitro* transcription/translation vector TP7-NF-ATp, which contains a cDNA fragment encoding the Rel domain of murine NF-ATp. The c-Jun expression vector, pGEM-c-Jun, was constructed by inserting a full-length cDNA of murine c-Jun into the PstI site of pGEM4. 1 µg of each plasmid DNA was transcribed from the T7 promoter and translated in rabbit reticulocyte lysate by using the TnT Coupled Transcription/Translation Kit (Promega, Madison, WI).

Electrophoretic mobility shift assays (EMSA) were performed as follows. 100 ng of double-stranded oligonucleotides were end-labeled with γ-^{32}P-dATP (DuPont NEN Research Product, Wilmington, DE) using T4 polynucleotide kinase (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ). The labeled ds-oligonucleotides were fractionated on 15-20% polyacrylamide gels, eluted overnight at 37° C in 1X TE and precipitated in ethanol. Binding assays were performed at room temperature for 20 minutes using 0.5 µg of recombinant proteins or 4 µl of *in vitro* translated products, 500 ng poly(dI-dC), and 20,000 cpm of probe in a 15 µl volume of 20 mM HEPES (pH 7.9), 100 mM KCl, 5% glycerol, 1mM EDTA, 5mM DTT, 0.1% NP-40, and 0.5 mg/ml BSA. The samples were then fractionated in 4% non-denaturing polyacrylamide gel containing 0.5X TBE at room temperature.

Oligonucleotides derived from the murine IL4 promoter used in EMSA were:

-59 to -27: 5'-CTCATTTTCCCTTGGTTTCAGCAACTTTAACTC-3' (SEQ ID NO: 1);

-79 to -60: 5'-ATAAAATTTTCCAATGTAAA-3' (SEQ ID NO: 2); and

-88 to -61: 5'-TGGTGTAATAAAATTTTCCAATGTAAA-3' (SEQ ID NO: 3).

The sequence of the MARE oligonucleotide used in EMSA was:

5'-GGAATTGCTGACTCAGCATTACT-3'(SEQ ID NO: 4).

All oligonucleotides were annealed with their respective reverse-complementary strands to form double-stranded oligonucleotides.

The results of EMSA with recombinant c-Maf are shown in Figure 6. The recombinant c-Maf protein bound well to both a consensus MARE oligonucleotide and to a 33 bp oligonucleotide containing the NF-AT site and MARE present in the IL-4 promoter. Binding was specifically competed by unlabeled homologous but not control probe. Further, c-Maf did not bind to an oligonucleotide containing only the NF-AT target sequence to which recombinant NF-ATp bound well. The ability of c-Maf to bind to the IL-4 promoter probe was specific since *in vitro* translated c-Jun protein did not bind to this oligonucleotide. The c-Jun protein was functional since it could bind to the consensus MARE which contains a core TRE site. These results indicate that c-Maf, but not another AP-1 family member (c-Jun), can bind to the MARE site within the proximal IL-4 promoter.

NF-AT proteins interact cooperatively with AP-1 family member proteins to form higher mobility complexes on IL-2 and IL-4 promoter DNA on EMSA (Jain, J. (1993) *Nature* 365:353-355; Rooney, J. *et al.* (1995) *Immunity* 2:545-553). That NF-AT proteins might interact with c-maf was suggested by the functional studies described in the previous examples. To determine if c-Maf interacted with NF-AT in the presence of DNA, recombinant NF-ATp and c-Maf proteins were used separately or together in EMSA with the 33 bp oligonucleotide containing both the NF-AT and adjacent MARE sites. The results are shown in Figure 6. Each protein alone bound to IL-4 promoter DNA. Recombinant c-Maf plus recombinant NF-ATp protein produced these complexes and in addition formed a higher mobility complex. No higher mobility complex was observed when c-Jun and NF-ATp proteins were used, consistent with the failure of c-Jun to bind this site. These results indicate that c-Maf can specifically bind *in vitro* to a sequence located in the proximal IL-4 promoter, previously shown to be functionally critical in Th2 cells, and that, like other AP-1 proteins, c-Maf can interact *in vitro* with NF-AT proteins.

EXAMPLE 7: The Ability of c-Maf to Transactivate the IL-4 Promoter Maps to the MARE and Th-2 Specific Footprint

An essential region of the IL-4 promoter located immediately upstream of the TATA element has been characterized by high resolution mutagenesis (Hodge, M. *et al.* (1995) *J. Immunol.* 154:6397-6405). Mutagenesis of this 33 bp region (-59 to -28) demonstrated multiple sites required for inducible IL-4 transcription in Th2 cells. These sites included an NF-AT target sequence, the region footprinted by Th2 extracts, and what is now recognized as a MARE. A series of IL-4 reporter gene constructs comprising 4 base pair linker-scanning mutants generated across this region were used to map the target sequence utilized by c-Maf *in vivo* in M12 cells. These cells were cotransfected with the c-maf expression vector and this series of mutant IL-4 promoter constructs. The results are shown in Figure 7A. Mutation of the MARE (muts 3 and 4), or the site defined by the Th2 footprint (mut 2), abrogated (muts 2 and 4) or partially abrogated (mut 3) the ability of transfected c-maf to drive IL-4 transcription. A modest effect in reducing c-maf transactivation was also observed for mutant 8 which disrupts the NF-AT sequence, consistent with the presence in M12 cells of endogenous NF-ATp and with the synergy between NF-ATp and c-maf demonstrated in the previous examples. Mutants 6 and 7 had no significant effect while mutant 5 had enhanced transactivation ability, consistent with previous observations in Th2 cells (Hodge, M. *et al.* (1995) *J. Immunol.* 154:6397-6405). The transactivation data is consistent with EMSA performed with recombinant c-Maf protein using as probe an oligonucleotide which contains this 33 bp region, and this same series of mutant oligonucleotides as cold competitors. The results of these EMSA experiments are shown in Figure 7B. These experiments indicate that c-Maf specifically binds to and transactivates the MARE in the proximal IL-4 promoter and that the adjacent Th2-specific element is intimately involved in both the binding and function of c-Maf.

EXAMPLE 8: Isolation of a NIP45 cDNA Using a Yeast Two-Hybrid Interaction Trap Assay

A yeast two-hybrid interaction trap assay was used to isolate proteins that could directly bind to the RHD of NF-ATp. An NF-ATp(RHD)-Gal4 fusion protein was prepared for use as the "bait" in the yeast two-hybrid assay by cloning a 900 bp fragment of murine NF-ATp (McCaffrey, P.G. *et al.* (1993) *Science* 262:750-754), spanning amino acids 228 to 520, into the BamHI site of vector pEG202 (Gyuris, J. *et al.* (1993)

Cell 75:791-803). In frame fusion of the NF-AT(p) polypeptide sequences to the Gal4 sequences was confirmed by DNA sequence analysis. This bait was used to screen a cDNA library prepared from the murine T cell line D10, constructed in the plasmid pJG4-5, to select for clones encoding polypeptides that interacted with the bait, using methodologies known in the art (see Gyuris, J. *et al.* (1993) *Cell* 75:791-803).

One class of interactors encoding a fusion protein with apparently high affinity for the NF-ATp(RHD)-Gal4 bait, as exhibited by high level of β-galactosidase activity and ability to confer leucine prototrophy, was isolated and termed NIP45 (NF-AT Interacting Protein 45). Figure 8 shows a photograph of yeast colonies (three representatives for each plasmid combination), cotransformed with the NIP45 plasmid and either the NF-ATp-RHD bait or control baits (Max-Gal4, CDK2-Gal4 and the control vector pEG202, expressing only an epitope tagged Gal4 protein), together with the LacZ reporter plasmid pSH18. The yeast colonies had been selected on appropriate media and were spotted onto plates containing Xgal and the nonrepressing carbon source galactose. Yeast colonies cotransformed with the NIP45 plasmid and the NF-ATp-RHD bait were blue in color, demonstrating expression of the LacZ reporter plasmid (indicative of NIP-45/NF-ATp-RHD interaction), whereas yeast colonies transformed with the NIP45 plasmid and the control baits were white in color, indicating no interaction of NIP45 with the control baits. Transformants were also tested on galactose containing media lacking leucine, and only those containing the NIP45 plasmid and the NF-ATp-RHD bait grew, further indicating the specific interaction of NIP45 with NF-ATp-RHD. The NIP45 cDNA isolated by the two-hybrid assay was a 1.9 kb DNA fragment.

EXAMPLE 9: Interaction of NIP45 and NF-ATp *In vivo* in Mammalian Cells

The ability of the NIP45 polypeptide to interact specifically with NF-ATp *in vivo* was tested in mammalian cells. The 1.9 kb NIP45 cDNA insert selected in the yeast two-hybrid system (described in Example 8) was subcloned into a mammalian expression vector which fuses the coding region to an epitope tag from a influenza hemagglutinin (HA) peptide, vector pCEP4-HA (Herrscher, R.F. *et al.* (1995) *Genes Dev.* 9:3067-3082), to create the expression vector NIP45-HA. This tagged construct was then cotransfected with an NF-ATp expression plasmid into HepG2 cells (which express low levels of NF-ATp). As controls, HepG2 cells also were cotransfected with NIP45-HA along with the parental expression vector for the NF-ATp construct (*i.e.*, the expression vector without the NF-ATp insert) or with the NF-ATp expression vector

along with an out of frame fusion of NIP45 with the epitope tag. Lysates were prepared from the transfected cells and immunoprecipitated with anti-NF-ATp antibody. Western blot analysis was then performed on the immunprecipitated material using either anti-NF-ATp or anti-HA antibodies.

The results of this experiment are shown in Figure 9. Western blot analysis of these samples using an HA-specific monoclonal antibody (mAb) demonstrated that the anti-NF-ATp antibody used for immunoprecipitation coimmunoprecipitated the HA-tagged NIP45 polypeptide. The lane showing transfection with only NIP45-HA (middle lane) reveals the low endogenous level of NF-ATp present in these cells. The amount of HA-tagged NIP45 protein immunoprecipitated was further increased by cotransfection with the NF-ATp expression plasmid demonstrating the specificity of this interaction (right lane). Western blot analysis of untreated lysates demonstrated that equivalent levels of NIP45-HA polypeptide were expressed in the samples tested for coimmunoprecipitation of NIP45-HA anti-NF-ATp antibodies. Furthermore, no immunoreactive material for either NF-ATp or the HA tagged protein was detected when performing immunoprecipitation using normal rabbit serum. These experiments demonstrate that NF-AT and NIP45 physically associate *in vivo* in mammalian cells.

<u>EXAMPLE 10</u>: Structural Analysis of NIP45 cDNAs

The 1.9 kb NIP45 cDNA insert from the clone isolated using the two-hybrid assay (described in Example 1) was used to screen a D10.G4 T cell lambda zap II cDNA library (Stratagene) to identify full length clones. Screening of a library containing approximately 8x 10^5 clones yielded 7 hybridizing clones most of which did not extend as far towards the 5' end as the original isolate. Sequence analysis of the longest clone (2.8 kb), however, demonstrated identity to the original clone at the 5' end. The structures of the original 1.9 kb cDNA isolate and the longest 2.8 kb cDNA isolate are compared in Figure 10. The 2.8 kb cDNA isolate contained an additional segment of 180 bp located 868 bp downstream from the 5' end of the original clone. Junction sequences at the ends of this 180 nucleotide segment indicate it to be an unspliced intron and conceptual translation of the nucleotide sequence within this region revealed an in-frame stop codon. Much of the additional sequence in this clone was at the 3' end and represented an extensive 3' untranslated region followed by a poly-A+ tail (see Figure 10). Such extensive 3' untranslated regions have been observed in many genes. Allowing for the splicing of the small intron and translation of the single large open

reading frame, the 2.8 kb cDNA clone is predicted to encode an identical polypeptide to that of the original 1.9 kb isolate.

The nucleotide and predicted amino acid sequences of the 1.9 kb cDNA isolate are shown in Figure 11 (and in SEQ ID NOs: 5 and 6, respectively). The coding region is shown from the first initiation codon through the first in frame stop codon. The nucleotide and amino acid positions are indicated to the right of the primary sequence. Conceptual translation of the 1.9 kb nucleotide sequence predicted a polypeptide of 412 amino acids with a molecular mass of 45 Kd, and hence the protein has been termed NF-AT Interacting Protein 45 (NIP45). Inspection of the amino acid sequence of NIP45 revealed a highly basic domain at the N-terminus, in which 13 of 32 amino acid are basic. This region is underlined in Figure 11. This basic region appears as a hydrophilic stretch in the hydrophobicity plot shown in Figure 12.

EXAMPLE 11: Tissue Expression of NIP45 mRNA

Northern blot analysis of RNA from different murine tissues was performed to investigate the tissue expression of NIP45 mRNA. 10 μg of total RNA from various tissues was separated on denaturing agarose gels, blotted and hybridized with a radiolabelled 1.4 kb NIP45 cDNA fragment. Samples were controlled for equivalent loading of RNA by comparison of ethidium bromide fluorescence. The results of the Northern blot analysis are shown in Figure 13. The hybridizations revealed a transcript of approximately 3.1 kb, which is of comparable size to the longest cDNA clones. RNA from testis contained an additional 1.4 Kb hybridizing species. The highest levels of NIP45 transcripts were seen in spleen, thymus and testis. The preferential expression in lymphoid organs may indicate a specific function for NIP45 in the immune system. The low intensity hybridization signal and the rare occurrence of NIP45 cDNA clones in the T cell cDNA library indicate that the NIP45 RNA is a relatively rare message.

EXAMPLE 12: Subcellular Localization of NIP45

Subcellular localization of epitope tagged NIP45 protein was determined by indirect immunofluorescence. BHK cells were transfected with 1 μg of an expression construct encoding an HA-epitope tagged NIP45 (pCEP4-HA), using methodologies known in the art (see Heald, R. *et al.* (1993) *Cell* 74:463-474). Transfected cells were incubated overnight, fixed, permeabilized as described (Heald, R. *et al.* (1993) *supra*) and probed with an anti-HA mAb 12CA5 (Boehringer Mannheim) plus

indocarbocyanine labelled donkey anti-mouse antibody (Jackson ImmunoResearch) and then counterstained with the dye Hoechst 33258. The results are shown in Figures 14A-B. Nuclear staining of NIP45 was observed with the indocarbocyanine labelled secondary reagent (see Figure 14A) by comparison to the same cells counterstained with the DNA staining dye Hoechst 33258 (see Figure 14B). The fluorescence pattern indicates that NIP45 is evenly distributed throughout the nucleus. Furthermore, this pattern matched that seen for cells transfected with NF-AT4 and stimulated with ionomycin (Shibasaki, F. *et al.* (1996) *Nature* 382:370-373; see also below). Stimulation with PMA and/or ionomycin did not affect the subcellular localization of this NIP45.

Control experiments were also performed on BHK cells transfected with NF-AT4. Cells were incubated overnight in culture media and either fixed directly or first stimulated with 1 mM ionomycin for 10 minutes before fixation and then processed as described above. The results are shown in Figures 14C-F. Unstimulated (Figs. 14C and 14D) or ionomycin treated (Figs. 14E and 14F) NF-AT4 transfectants were probed with an anti-NF-AT4 specific antibody followed by a indocarbocyanine labelled secondary reagent and Hoechst 33258. Indocarbocyanine fluorescence demonstrates the pattern of staining for cytoplasmic localized NF-AT4 in unstimulated transfectants (Fig. 14C) and nuclear localized NF-AT4 in stimulated cells (Fig. 14E). Adjacent panels (Fig. 14D and 14F, respectively) show the same field exposed for detection of nuclei by staining with Hoechst 33258.

The effect of NIP45 on the nuclear translocation of NF-AT4 also was investigated. HepG2 cells were transfected with either NF-AT4 or NF-AT4 plus NIP45 and stimulated the following day with 1 μM ionomycin for 0, 2, 4, 8 or 15 minutes. For one sample, the cells were stimulated for 15 minutes with ionomycin and then washed with fresh media and allowed to rest for an additional 15 minutes (indicated as "15 min. + 15 min. rest" in Table 1). This analysis is designed to examine the function of NIP45 as a nuclear retention factor. Fifteen minutes has been shown to be sufficient time for NF-AT4 to be exported to the cytoplasm (Shibasaki, F. *et al.* (1996) *Nature* 382:370-373). All samples were then fixed and analyzed by immunoflourescence for translocation of NF-AT4 as described above. The results are summarized below in Table 2. Subcellular localization of NF-AT4 in the cytoplasm is indicated by a (-) and nuclear translocation of NF-AT4 is indicated by (+).

Table 2: Nuclear Translocation of NF-AT4

Time	Ionomycin	Ionomycin + NIP45
0 min.	-	-
2 min.	+/-	+/-
4 min.	+/-	+/-
8 min.	+	+
15 min.	+	+
15 min. + 15 min. rest	-	-

No difference in the rate of nuclear import or export of NF-AT4 was observed in the presence of NIP45, indicating that nuclear trafficking of NF-AT4 in response to changes in intracellular calcium levels was not affected by the overexpression of exogenous NIP45.

EXAMPLE 13: Functional Activity of NIP45 in Regulating Gene Expression

To test for a functional role of NIP45 in NF-AT-driven transcription, NIP45 was expressed at high levels in HepG2 cells. HepG2 cells were chosen because they have low levels of endogenous NF-AT, and ectopic expression of NF-AT family member proteins has been shown to transactivate NF-AT-driven transcription in this cell line in the absence of exogenous stimulation (Hoey, T. *et al.* (1995) *Immunity* 2:461-472). HepG2 cells were transfected with a 3X NF-AT-CAT reporter from the IL-2 gene (Venkataraman, L. *et al.* (1994) *Immunity* 1:189-196) and control or expression plasmids for a NIP45 and NF-AT family members (NF-ATp, NF-ATc, NF-AT3, NF-AT4). HepG2 cells were transfected by the DEAE-Dextran method as described in Hoey, T. *et al.* (1995) *supra*, and CAT assays were performed according to standard methodologies. The results are shown in Figure 15. One representative assay for each combination is shown adjacent to a bar graph representing relative CAT activity for each group. Fold induction was calculated by normalizing the CAT activity of cells transfected with the CAT reporter and each parental expression vector to one. Values represent the relative level of CAT expression above this control transfection. All transfections were performed at least three times with one representative autoradiograph shown.

Transfection of NIP45 alone into HepG2 cells with a 3X NF-AT-CAT reporter did not lead to a significant increase in CAT expression demonstrating that NIP45 cannot act on its own to transactivate an NF-AT target sequence. Overexpression of NF-ATp alone resulted in substantial (6-fold over vector control) transactivation of the

NF-AT-CAT reporter, consistent with previous reports (Hoey, T. *et al.* (1995) *supra*). Cotransfection of NIP45 plus NF-ATp resulted in a 4-5 fold increase in CAT activity relative to transfection with NF-ATp alone and a 25-30 fold increase over that seen with vector alone. This increase was not observed when a mutant 3X NF-AT-CAT reporter or a control MHC class II promoter reporter was used thus demonstrating its target site specificity. To confirm that the polypeptide product encoded by the NIP45 cDNA was responsible for this enhanced transactivation, a frame shift mutation was introduced in the coding region by creating a two base deletion at nucleotide 50. This alteration results in the introduction of missense mutations at amino acid 13 and termination of the polypeptide after an additional 22 residues. Assays using this NIP45Δ construct demonstrated its failure to transactivate the NF-AT reporter in the presence or absence of NF-ATp thus confirming that the enhanced transactivation observed was due to the polypeptide expressed from NIP45 cDNA. Transactivation experiments were also performed in the B cell line M12 and the T cell clone D10 with similar although less dramatic results, which may be due to higher levels of endogenous NIP45 or NF-ATp in these latter cell lines. These experiments demonstrate that NIP45 substantially and specifically potentiates transcription induced by NF-ATp, an activity that requires interaction with NF-ATp.

NF-AT proteins share approximately 70% identity within the RHD, raising the possibility that NIP45 could also interact with other NF-AT family members. To test this, NIP45 was cotransfected as above with expression constructs encoding either NF-ATc, NF-AT3 or NF-AT4 plus the 3X NF-AT-CAT reporter plasmid. The results of these experiments are also shown in Figure 8. It has previously been demonstrated that all NF-AT family members can transactivate a reporter gene containing 3 copies of an NF-AT/AP1 site when overexpressed in HepG2 cells, although to different levels (Hoey, T. *et al.* (1995) *supra*). In the absence of NIP45, NF-ATp was the most potent transactivator of the NF-AT-CAT reporter followed by NF-ATc and NF-AT3 with only weak transactivation by NF-AT4, consistent with previous data (McCaffrey, P.G. *et al.* (1993) *Science* 262:750-754). When NF-ATc, NF-AT3 or NF-AT4 were cotransfected with NIP45, NIP45 substantially potentiated both NF-ATc and NF-AT3-driven transactivation and weakly potentiated NF-AT4-mediated transactivation (Figure 15). Cooperation with NF-ATc in HepG2 cells is consistent with the observation that NIP45 interacts with an NF-ATc RHD bait in yeast cells. Overall, NIP45 overexpression resulted in a 4-fold increase in transactivation by NF-ATc, a 3-fold increase in NF-AT3-driven transactivation and a 2-fold increase in NF-AT4-driven transcription. The ability of NIP45 to potentiate the activity of all NF-AT family members is not surprising given

the high degree of sequence conservation of the RHD of the NF-AT family members. A sequence comparison of the NF-AT RHD domains reveals a higher level of sequence identity in the amino terminal portion compared to that of the carboxyl terminus (Hoey, T. *et al.* (1995) *supra*). Thus it is likely that the NIP45/NF-AT interaction site is located in the 5' portion of the RHD.

Although a reporter construct containing multiple copies of the NF-AT binding site provides a sensitive method for measuring transactivation by NF-AT and NIP45, we sought to determine if NIP45 was functional in the context of a native NF-AT-dependent promoter. IL-4 expression is highly tissue specific and restricted to the Th2 subset of T cells and to mast cells. The IL-4 promoter contains multiple NF-AT binding sites which have been shown to be critical for expression of IL-4 (Rooney, J.W. *et al.* (1995) *Immunity* 2:473-483). Furthermore, the proto-oncogene c-Maf has been shown to direct tissue specific expression of IL-4 (Examples 3 and 4). Thus, the IL-4 promoter is not active in the HepG2 cell line but can be activated by the introduction of NF-ATp and c-Maf. In cotransfection experiments carried out as described above, HepG2 cells were transfected with an IL-4-CAT reporter construct (extending to -732 bp of the IL-4 promoter) and expression vectors or controls for NIP45, NF-ATp and c-Maf. The controls for NIP45 was a frame shift mutant at amino acid 13. Controls for NF-ATp and c-Maf were the empty expression vectors pREP4 and pMEX respectively (Ho, I.C. *et al.* (1996) *Cell* 85:973-983). The results of these experiments are shown in Figure 16 (representative CAT assays and bar graphs are depicted as in Figure 15). The data indicate that introduction of NIP45 together with NF-ATp and c-Maf results in an additional 9-fold increase in the activity of the IL-4 promoter relative to that seen for NF-ATp and c-Maf alone. NIP45 also increased the activity of the IL-4 promoter in the absence of transfected NF-ATp, an effect likely due to interaction with endogenous NF-ATp.

EXAMPLE 14: Transient Overexpression of NIP45 with NF-ATp and c-Maf Results in Endogenous IL-4 Production

To determine whether the combination of NIP45, NF-ATp and c-Maf was sufficient to induce endogenous IL-4 expression by cells that do not normally produce IL-4, M12 B lymphoma cells were transiently cotransfected with expression plasmids for NF-ATp and c-Maf together with NIP45 or pCI vector control. M12 cells were transiently transfected by electroporation as previously described (Ho, I.C. *et al.* (1996) *Cell* 85:973-983) by

incubating 3 x 10^6 cells in 0.4 ml of PBS with 5 μg of each plasmid for 10 minutes at room temperature prior to electroporation at 975 μF, 280 V. Levels of IL-4 in the supernatants harvested 72 hours later were measured by a commercially available IL-4 ELISA (Pharmingen), performed according to the manufacturer's instructions except with modification as described (Ho, I.C. *et al.* (1996) *supra*). Four independent sets of transient transfections were done and assayed for secretion of IL-4 into the culture supernatant. Results from a representative experiment from one of the four independent transfections is shown in Figure 17. For each set of transfections, inclusion of NIP45 led to a dramatic increase in IL-4 production. Cells transfected with NIP45 produced 50-200 fold more endogenous IL-4 than cells that did not receive NIP45, in which IL-4 production was near the limit of detection.

<u>EXAMPLE 15</u>: Expression of p18 mRNA is Downregulated During T Helper Cell Differentiation *In vitro*

The Maf family protein p18 is a member of the "small mafs" that lack the amino terminal two thirds of c-Maf that contains the transactivating domain. To examine the expression of p18 transcripts during differentiation of normal T helper cells to the Th2 phenotype, *in vitro* differentiation experiments were performed as described above in Example 2. Naive spleen cells (Th precursor (Thp) cells) were driven along a Th1 or Th2 pathway by treatment with anti-CD3 in the presence of cytokines and anti-cytokine antibodies (IFNγ and anti-IL-4 for Th1, IL-4 and anti-IFNγ for Th2). Northern blot analysis of differentiating cells harvested at various time points after stimulation (day 0, 1, 3, 5, or 7) were performed to analyze the expression of p18 and *c-maf* transcripts. The results for *c-maf* and p18 expression *in vitro* differentiated Th2 cells are shown in Figure 18. Consistent with results described above, expression of the *c-maf* transcript was low level or undetectable at day 0 but increased as the cells differentiated along the Th2 pathway. In contrast, expression of p18 transcript was detectable at day 0 (*i.e.*, in undifferentiated T cells) but decreased to essentially undetectable levels as the cells differentiated along the Th2 pathway. These results indicate that p18 expression is downregulated in normal T helper cells during differentiation to the Th2 phenotype.

<u>EXAMPLE 16</u>: p18 Represses IL-4 Promoter Activity

To examine whether p18 expression affects IL-4 promoter activity, cotransfection experiments were performed in M12 B lymphoma cells. Methodologies

used for these experiments were as described above in Example 3. An IL-4 promoter/CAT reporter gene construct was transfected into M12 cells with either a c-Maf expression vector, a p18 expression vector or both c-Maf and p18 expression vectors. Representative results of CAT assays are shown in Figure 19. Expression of c-Maf alone (5 μg of plasmid) resulted in activation of the IL-4 promoter construct (see lane 2 of Figure 19), evidenced by detectable CAT activity in the M12 cells. Coexpression of the p18 expression vector (2.5, 5 or 10 μg) with c-Maf resulted in decreased CAT activity (see lanes 3, 4, and 5 of Figure 19), with increasing amounts of p18 leading to greater decreases in the observed CAT activity. Expression of p18 alone in the M12 cells did not result in detectable CAT activity in the cells (see lane 6 of Figure 19). These results demonstrate that p18 can repress IL-4 promoter activity that is stimulated by c-Maf.

EXAMPLE 17: Transgenic Mice that Overexpress c-Maf

This example describes overexpression of c-Maf protein in T cells of transgenic mice. A schematic diagram of the c-maf transgenic construct is shown in Figure 20. This construct comprises the 4 kb mouse c-maf cDNA and the first intron of the mouse c-maf gene. Expression of the c-maf cDNA is controlled by the CD4 promoter/enhancer regulatory region, which is operatively linked to the 5' end of the c-maf first intron, thereby conferring T cell-specific expression on the construct. An SV40 polyadenylation site is linked to the 3' end of the c-maf cDNA. This c-maf transgenic construct was microinjected into fertilized mouse oocytes and transgenic mice were prepared according to standard procedures.

The phenotype of the c-maf transgenic animals were compared to that of wild type animals using a number of different assays. First, total numbers of cells in lymphoid organs (lymph nodes, spleen and thymus) were quantitated, the results of which are shown in Figure 21. This experiment demonstrated that the c-maf transgenic mice exhibited decreased cell numbers in all three lymphoid organs examined compared to wild type mice. Next, the abundance of specific thymocyte populations was compared in c-maf transgenic and wild type mice, using flow cytometry. Double positive ($CD4^+CD8^+$), double negative ($CD4^-CD8^-$), and single positive thymocytes ($CD4^+$ or $CD8^+$) were quantitated. The ratio of CD4 to CD8 cells (CD4/CD8) also was determined. The results are summarized below in Table 3.

Table 3: Abundance of Specific Thymocyte Populations in c-Maf Transgenic Mice

	$CD4^-CD8^-$	$CD4^+CD8^+$	$CD4^+$	$CD8^+$	CD4/CD8*
Wild type	4.2 ± 0.6	71.6 ± 9.5	8.5 ± 1.6	2.8 ± 0.6	3.2 ± 0.8
Transgenic	2.8 ± 0.6	11.8 ± 8.7	3.8 ± 0.8	2.0 ± 0.6	2.0 ± 0.2

These results demonstrate that c-maf transgenic mice have a dramatic decrease in the number of double positive thymocytes compared to wild type mice. Moreover, the c-maf transgenic mice have significantly decreased numbers of $CD4^+$ single positive thymocytes. Finally, the basal level of IgE in the serum of c-maf transgenic and wild type mice was quantiated. The results are shown in Figure 22. This experiment demonstrates that c-maf transgenic mice exhibit increased basal levels of serum IgE as compared to the wild type mice.

The phenotype of the c-maf transgenic mice described above (namely small spleen and thymus, decreased numbers of double positive thymocytes and single positive CD4+ thymocytes, and increased basal levels of serum IgE) is very similar to the phenotype described for IL-4 overexpressor transgenic mice (see Tepper et al. (1990) *Cell* 62:457; and Lewis et al. (1991) *J. Exp. Med.* 173:89).

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
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(ii) TITLE OF INVENTION: Methods and Compositions for Regulating T Cell Subsets by Modulating Transcription Factor Activity

(iii) NUMBER OF SEQUENCES: 6

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US
(B) FILING DATE:
(C) CLASSIFICATION:

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/636,602
(B) FILING DATE: 23-APR-1996
(C) CLASSIFICATION:

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/755,592
(B) FILING DATE: 25-NOV-1996
(C) CLASSIFICATION:

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/755,584
(B) FILING DATE: 25-NOV-1996
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
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(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (617)227-7400
(B) TELEFAX: (617)227-5941

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CTCATTTTCC CTTGGTTTCA GCAACTTTAA CTC 33

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ATAAAATTTT CCAATGTAAA 20

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TGGTGTAATA AAATTTTCCA ATGTAAA 27

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
GGAATTGCTG ACTCAGCATT ACT                                            23
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1946 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 13..1248

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
ACAGTGTGGG AG ATG GCG GAA CCA CTG AGG GGA CGT GGT CCG AGG TCC        48
              Met Ala Glu Pro Leu Arg Gly Arg Gly Pro Arg Ser
                1               5                  10

CGC GGT GGC CGA GGC GCT CGG AGA GCC CGA GGC GCC CGT GGC CGG TGT      96
Arg Gly Gly Arg Gly Ala Arg Arg Ala Arg Gly Ala Arg Gly Arg Cys
        15                  20                  25

CCT CGC GCC CGG CAG TCT CCG GCT AGG CTC ATT CCA GAC ACC GTG CTT     144
Pro Arg Ala Arg Gln Ser Pro Ala Arg Leu Ile Pro Asp Thr Val Leu
    30                  35                  40

GTG GAC TTG GTC AGT GAC AGC GAC GAA GAG GTC TTG GAA GTC GCA GAC     192
Val Asp Leu Val Ser Asp Ser Asp Glu Glu Val Leu Glu Val Ala Asp
45                  50                  55                  60

CCA GTA GAG GTG CCG GTC GCC CGC CTC CCC GCG CCG GCT AAA CCT GAG     240
Pro Val Glu Val Pro Val Ala Arg Leu Pro Ala Pro Ala Lys Pro Glu
                65                  70                  75

CAG GAC AGC GAC AGT GAC AGT GAA GGG GCG GCC GAG GGG CCT GCG GGA     288
Gln Asp Ser Asp Ser Asp Ser Glu Gly Ala Ala Glu Gly Pro Ala Gly
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                80                  85                  90

GCC CCG CGT ACA TTG GTG CGA CGG CGG CGG CGG CGG CTG CTG GAT CCC      336
Ala Pro Arg Thr Leu Val Arg Arg Arg Arg Arg Arg Leu Leu Asp Pro
         95                 100                 105

GGA GAG GCG CCG GTG GTC CCA GTG TAC TCC GGG AAG GTA CAG AGC AGC      384
Gly Glu Ala Pro Val Val Pro Val Tyr Ser Gly Lys Val Gln Ser Ser
    110                 115                 120

CTC AAC CTC ATT CCA GAT AAT TCA TCC CTC TTG AAA CTG TGC CCT TCA      432
Leu Asn Leu Ile Pro Asp Asn Ser Ser Leu Leu Lys Leu Cys Pro Ser
125                 130                 135                 140

GAG CCT GAA GAT GAG GCA GAT CTG ACA AAT TCT GGC AGT TCT CCC TCT      480
Glu Pro Glu Asp Glu Ala Asp Leu Thr Asn Ser Gly Ser Ser Pro Ser
                145                 150                 155

GAG GAT GAT GCC CTG CCT TCA GGT TCT CCC TGG AGA AAG AAG CTC AGA      528
Glu Asp Asp Ala Leu Pro Ser Gly Ser Pro Trp Arg Lys Lys Leu Arg
            160                 165                 170

AAG AAG TGT GAG AAA GAA GAA AAG AAA ATG GAA GAG TTT CCG GAC CAG      576
Lys Lys Cys Glu Lys Glu Glu Lys Lys Met Glu Glu Phe Pro Asp Gln
        175                 180                 185

GAC ATC TCT CCT TTG CCC CAA CCT TCG TCA AGG AAC AAA AGC AGA AAG      624
Asp Ile Ser Pro Leu Pro Gln Pro Ser Ser Arg Asn Lys Ser Arg Lys
    190                 195                 200

CAT ACG GAG GCG CTC CAG AAG CTA AGG GAA GTG AAC AAG CGT CTC CAA      672
His Thr Glu Ala Leu Gln Lys Leu Arg Glu Val Asn Lys Arg Leu Gln
205                 210                 215                 220

GAT CTC CGC TCC TGC CTG AGC CCC AAG CAG CAC CAG AGT CCA GCC CTT      720
Asp Leu Arg Ser Cys Leu Ser Pro Lys Gln His Gln Ser Pro Ala Leu
                225                 230                 235

CAG AGC ACA GAT GAT GAG GTG GTC CTA GTG GAA GGG CCT GTC TTG CCA      768
Gln Ser Thr Asp Asp Glu Val Val Leu Val Glu Gly Pro Val Leu Pro
            240                 245                 250

CAG AGC TCT CGA CTC TTT ACA CTC AAG ATC CGG TGC CGG GCT GAC CTA      816
Gln Ser Ser Arg Leu Phe Thr Leu Lys Ile Arg Cys Arg Ala Asp Leu
        255                 260                 265

GTG AGA CTG CCT GTC AGG ATG TCG GAG CCC CTT CAG AAT GTG GTG GAT      864
Val Arg Leu Pro Val Arg Met Ser Glu Pro Leu Gln Asn Val Val Asp
    270                 275                 280

CAC ATG GCC AAT CAT CTT GGG GTG TCT CCA AAC AGG ATT CTT TTG CTT      912
His Met Ala Asn His Leu Gly Val Ser Pro Asn Arg Ile Leu Leu Leu
285                 290                 295                 300

TTT GGA GAG AGT GAA CTG TCT CCT ACT GCC ACC CCT AGT ACC CTA AAG      960
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Phe Gly Glu Ser Glu Leu Ser Pro Thr Ala Thr Pro Ser Thr Leu Lys
                305                 310                 315

CTT GGA GTG GCT GAC ATC ATT GAT TGT GTG GTG CTA GCA AGC TCT TCA    1008
Leu Gly Val Ala Asp Ile Ile Asp Cys Val Val Leu Ala Ser Ser Ser
            320                 325                 330

GAG GCC ACA GAG ACA TCC CAG GAG CTC CGG CTC CGG GTG CAG GGG AAG    1056
Glu Ala Thr Glu Thr Ser Gln Glu Leu Arg Leu Arg Val Gln Gly Lys
        335                 340                 345

GAG AAA CAC CAG ATG TTG GAG ATC TCA CTG TCT CCT GAT TCT CCT CTT    1104
Glu Lys His Gln Met Leu Glu Ile Ser Leu Ser Pro Asp Ser Pro Leu
    350                 355                 360

AAG GTT CTC ATG TCA CAC TAT GAG GAA GCC ATG GGA CTC TCT GGA CAC    1152
Lys Val Leu Met Ser His Tyr Glu Glu Ala Met Gly Leu Ser Gly His
365                 370                 375                 380

AAG CTC TCC TTC TTC TTT GAT GGG ACA AAG CTT TCA GGC AAG GAG CTG    1200
Lys Leu Ser Phe Phe Phe Asp Gly Thr Lys Leu Ser Gly Lys Glu Leu
                385                 390                 395

CCA GCT GAT CTG GGC CTG GAA TCC GGA GAT CTC ATC GAA GTC TGG GGC    1248
Pro Ala Asp Leu Gly Leu Glu Ser Gly Asp Leu Ile Glu Val Trp Gly
            400                 405                 410

TGAAGCTCTC ACCCTGTTCG GACGCAAAGC CAAGACATGG AGACAATAGC TCCCAATTTT  1308

ATTATTGTGA TTTTTCGCCC CATAAGGGCT AACAGAAACT GAATTAGAAC TTGTTTACTT  1368

ATTTATTTCT GGTGCTGGGG ATTGAACCCC AGACTATGCA CATGCTAAGG ATGTATGAAG  1428

TGGAGGCAAA ACCAAGGCAT TACCTTTAGC CAGCCTCTAG TAGACTGTAG TGTCAAGCAA  1488

GTGGCTACTT GGTAGTTGTG TGGCTCTGTG TATGTTTGTG CTGTATTTGG CAGCCCCTGG  1548

GGCACATAGA AGGGACCTTG GCTTCCCTAC CATTTCACGT TCGCTGGTGC CCTTTCCTTC  1608

ATCAGATGAC TTCTGTGAAG CTGCCTATGT TGAGTGTGTT GAACTAAATG AGCTCTGCTT  1668

TGGGTGTCCA GGCCTGGGGT TTGTGCCGCA GTTGGAGCCA GCAGTGACTT CACTCTGACT  1728

TGGGACTGAG AATGCATTTC CTGGTGGAGA CACTCGGGTG CAGAAATATA ACAGAAGGTG  1788

ACATACATGC TGAAGCTGAG GACTAGGTCG AAAGTTAACG ACGTTGCATT TTCAGCCTTG  1848

GGTATCCTCT CTGCCTGCCA GGACTCTAGC CAGTGTCTGG TACACACTTC TTGGCATGGA  1908

CACCTAGGTC GACGCGGGCG CGATTCGGCC GACTCGAG                          1946
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 412 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
Met Ala Glu Pro Leu Arg Gly Arg Gly Pro Arg Ser Arg Gly Gly Arg
 1               5                  10                  15

Gly Ala Arg Arg Ala Arg Gly Ala Arg Gly Arg Cys Pro Arg Ala Arg
            20                  25                  30

Gln Ser Pro Ala Arg Leu Ile Pro Asp Thr Val Leu Val Asp Leu Val
        35                  40                  45

Ser Asp Ser Asp Glu Glu Val Leu Glu Val Ala Asp Pro Val Glu Val
    50                  55                  60

Pro Val Ala Arg Leu Pro Ala Pro Ala Lys Pro Glu Gln Asp Ser Asp
65                  70                  75                  80

Ser Asp Ser Glu Gly Ala Ala Glu Gly Pro Ala Gly Ala Pro Arg Thr
                85                  90                  95

Leu Val Arg Arg Arg Arg Arg Arg Leu Leu Asp Pro Gly Glu Ala Pro
            100                 105                 110

Val Val Pro Val Tyr Ser Gly Lys Val Gln Ser Ser Leu Asn Leu Ile
        115                 120                 125

Pro Asp Asn Ser Ser Leu Leu Lys Leu Cys Pro Ser Glu Pro Glu Asp
    130                 135                 140

Glu Ala Asp Leu Thr Asn Ser Gly Ser Ser Pro Ser Glu Asp Asp Ala
145                 150                 155                 160

Leu Pro Ser Gly Ser Pro Trp Arg Lys Lys Leu Arg Lys Lys Cys Glu
                165                 170                 175

Lys Glu Glu Lys Lys Met Glu Glu Phe Pro Asp Gln Asp Ile Ser Pro
            180                 185                 190

Leu Pro Gln Pro Ser Ser Arg Asn Lys Ser Arg Lys His Thr Glu Ala
        195                 200                 205

Leu Gln Lys Leu Arg Glu Val Asn Lys Arg Leu Gln Asp Leu Arg Ser
    210                 215                 220

Cys Leu Ser Pro Lys Gln His Gln Ser Pro Ala Leu Gln Ser Thr Asp
225                 230                 235                 240

Asp Glu Val Val Leu Val Glu Gly Pro Val Leu Pro Gln Ser Ser Arg
                245                 250                 255
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Leu Phe Thr Leu Lys Ile Arg Cys Arg Ala Asp Leu Val Arg Leu Pro
            260                 265                 270

Val Arg Met Ser Glu Pro Leu Gln Asn Val Val Asp His Met Ala Asn
        275                 280                 285

His Leu Gly Val Ser Pro Asn Arg Ile Leu Leu Leu Phe Gly Glu Ser
    290                 295                 300

Glu Leu Ser Pro Thr Ala Thr Pro Ser Thr Leu Lys Leu Gly Val Ala
305                 310                 315                     320

Asp Ile Ile Asp Cys Val Val Leu Ala Ser Ser Ser Glu Ala Thr Glu
                325                 330                 335

Thr Ser Gln Glu Leu Arg Leu Arg Val Gln Gly Lys Glu Lys His Gln
            340                 345                 350

Met Leu Glu Ile Ser Leu Ser Pro Asp Ser Pro Leu Lys Val Leu Met
        355                 360                 365

Ser His Tyr Glu Glu Ala Met Gly Leu Ser Gly His Lys Leu Ser Phe
    370                 375                 380

Phe Phe Asp Gly Thr Lys Leu Ser Gly Lys Glu Leu Pro Ala Asp Leu
385                 390                 395                     400

Gly Leu Glu Ser Gly Asp Leu Ile Glu Val Trp Gly
                405                 410
```

CLAIMS

We claim:

1. A method for modulating production of a T helper type 2 (Th2)-associated cytokine by a cell comprising contacting the cell with an agent that modulates the expression or activity of a transcription factor such that production of the Th2-associated cytokine by a cell is modulated, wherein the transcription factor cooperates with a Nuclear Factor of Activated T cells (NF-AT) family protein to regulate expression of the Th2-associated cytokine gene.

2. The method of claim 1, wherein the transcription factor is a Th2-specific transcription factor.

3. The method of claim 1, wherein the transcription factor is a maf family protein.

4. The method of claim 3, wherein the maf family protein is c-Maf.

5. The method of claim 3, wherein the maf family protein is a small maf protein.

6. The method of claim 5, wherein the small maf protein is p18.

7. The method of claim 1, wherein the transcription factor interacts with an NF-AT family protein.

8. The method of claim 7, wherein the transcription factor interacts with the Rel Homology Domain of an NF-AT family protein.

9. The method of claim 8, wherein the transcription factor is NIP45.

10. The method of any of claims 1-9, wherein the agent acts intracellularly to modulate the expression or activity of the transcription factor.

11. The method of any of claims 1-9, wherein the agent is a nucleic acid molecule encoding the transcription factor, wherein the nucleic acid molecule is introduced into the cell in a form suitable for expression of the transcription factor in the cell.

12. The method of any of claims 1-9, wherein the agent is an intracellular binding molecule.

13. The method of any of claims 1-9, wherein the cell is contacted with at least one additional agent that modulates the activity of at least one additional transcription factor that contributes to the regulation of the Th2-associated cytokine gene.

14. The method of claim 13, wherein the at least one additional transcription factor is selected from the group consisting of NF-AT family proteins, NF-AT-interacting proteins, maf family proteins and AP-1 family proteins.

15. The method of claim 11, wherein at least one additional nucleic acid molecule encoding at least one additional transcription factor that contributes to the regulation of the Th2-associated cytokine gene is introduced into the cell.

16. The method of claim 15, wherein the at least one additional transcription factor is selected from the group consisting of NF-AT family proteins, NF-AT-interacting proteins, maf family proteins and AP-1 family proteins.

17. The method of any of claims 1-9, wherein the cell is a T helper type 1 (Th1) cell, a B cell or a nonlymphoid cell.

18. The method of any of claims 1-9, wherein production of a Th2-associated cytokine by the cell is stimulated.

19. The method of any of claims 1-9, wherein production of a Th2-associated cytokine by the cell is inhibited.

20. The method of any of claims 1-9, wherein the Th2-associated cytokine is interleukin-4.

21. The method of any of claims 1-9, further comprising administering the cell to a subject to thereby modulate development of T helper type 1 (Th1) or T helper type 2 (Th2) cells in a subject.

22. A recombinant expression vector comprising a nucleotide sequence encoding a maf family protein operatively linked to regulatory sequences that direct expression of the maf family protein specifically in lymphoid cells.

23. The recombinant expression vector of claim 22, wherein the regulatory sequences direct expression of the maf family protein specifically in T cells.

24. The recombinant expression vector of claim 22, wherein the regulatory sequences direct expression of the maf family protein specifically in B cells.

25. A recombinant expression vector comprising a nucleotide sequence encoding a maf family protein operatively linked to regulatory sequences that direct expression of the maf family protein specifically in hematopoietic stem cells.

26. A host cell into which a recombinant expression vector encoding a maf family protein has been introduced, wherein the host cell is a lymphoid cell.

27. The host cell of claim 26, which is a T cell.

28. The host cell of claim 26, which is a B cell.

29. A host cell into which a recombinant expression vector encoding a maf family protein has been introduced, wherein the host cell is a hematopoietic stem cell.

30. An isolated nucleic acid molecule comprising a nucleotide sequence encoding NIP45 or a biologically active portion thereof.

31. An isolated nucleic acid molecule comprising a nucleotide sequence encoding a protein, wherein the protein comprises an amino acid sequence at least 60 % homologous to the amino acid sequence of SEQ ID NO: 6 and interacts with the Rel Homology Domain of an NF-AT family protein.

32. The isolated nucleic acid molecule of claim 31, wherein the protein comprises an amino acid sequence at least 70 % homologous to the amino acid sequence of SEQ ID NO: 6.

33. The isolated nucleic acid molecule of claim 31, wherein the protein comprises an amino acid sequence at least 80 % homologous to the amino acid sequence of SEQ ID NO: 6.

34. The isolated nucleic acid molecule of claim 31, wherein the protein comprises an amino acid sequence at least 90 % homologous to the amino acid sequence of SEQ ID NO: 6.

35. An isolated nucleic acid molecule at least 15 nucleotides in length which hybridizes under stringent conditions to a nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO: 5.

36. The isolated nucleic acid molecule of claim 35, which comprises a naturally-occurring nucleotide sequence.

37. The isolated nucleic acid molecule of claim 36, which encodes mouse NIP45.

38. The isolated nucleic acid molecule of claim 36, which encodes human NIP45.

39. An isolated nucleic acid molecule comprising the coding region of the nucleotide sequence of SEQ ID NO: 5.

40. The isolated nucleic acid molecule of claim 39, comprising the nucleotide sequence of SEQ ID NO: 5.

41. An isolated nucleic acid molecule encoding the amino acid sequence of SEQ ID NO: 6.

42. An isolated nucleic acid molecule encoding a NIP45 fusion protein.

43. An isolated nucleic acid molecule which is antisense to the nucleic acid molecule of claim 30.

44. An isolated nucleic acid molecule which is antisense to the coding strand of a nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO: 5.

45. The isolated nucleic acid molecule of claim 44, which is antisense to a coding region of the coding strand of the nucleotide sequence of SEQ ID NO: 5.

46. The isolated nucleic acid molecule of claim 44, which is antisense to a noncoding region of the coding strand of the nucleotide sequence of SEQ ID NO: 5.

47. A vector comprising the nucleic acid molecule of any of claims 30-46.

48. The vector of claim 47, which is a recombinant expression vector.

49. A host cell containing the vector of claim 47 or 48.

50. A method for producing NIP45 protein comprising culturing the host cell of claim 49 in a suitable medium until NIP45 protein is produced.

51. The method of claim 50, further comprising isolating NIP45 protein from the medium or the host cell.

52. An isolated NIP45 protein or a biologically active portion thereof.

53. An isolated protein which comprises an amino acid sequence at least 60 % homologous to the amino acid sequence of SEQ ID NO: 6 and interacts with the Rel Homology Domain of an NF-AT family protein.

54. The isolated protein of claim 53, which is at least 70% homologous to the amino acid sequence of SEQ ID NO: 6.

55. The isolated protein of claim 53, which is at least 80% homologous to the amino acid sequence of SEQ ID NO: 6.

56. The isolated protein of claim 53, which is at least 90% homologous to the amino acid sequence of SEQ ID NO: 6.

57. A fusion protein comprising a NIP45 polypeptide operatively linked to a non-NIP45 polypeptide.

58. An antigenic peptide of NIP45 comprising at least 8 amino acid residues of the amino acid sequence shown in SEQ ID NO: 6, the peptide comprising an epitope of NIP45 such that an antibody raised against the peptide forms a specific immune complex with NIP45.

59. An antibody that specifically binds NIP45 protein.

60. The antibody of claim 59, which is a monoclonal antibody.

61. The antibody of claim 59, which is coupled to a detectable substance.

62. A pharmaceutical composition comprising the antibody of any of claims 59-61 and a pharmaceutically acceptable carrier.

63. A nonhuman transgenic animal that contains cells carrying a transgene encoding NIP45 protein.

64. The nonhuman transgenic animal of claim 63, which contains cells having an altered endogenous NIP45 gene.

65. A nonhuman transgenic animal that contains cells carrying a transgene encoding a maf family protein.

66. The nonhuman transgenic animal of claim 65, wherein the maf family protein is expressed preferentially in T cells of the animal.

67. A method for identifying a compound that modulates the activity of a transcription factor that cooperates with a Nuclear Factor of Activated T cells (NF-AT) family protein to regulate expression of a Th2-associated cytokine gene, comprising

providing a indicator composition having activity of a transcription factor that cooperates with an NFAT family protein to regulate expression of the Th2-associated cytokine gene;

contacting the indicator composition with a test compound; and

determining the effect of the test compound on the activity of the transcription factor in the indicator composition to thereby identify a compound that modulates the activity of a transcription factor that cooperates with an NFAT family protein to regulate expression of a Th2-associated cytokine gene.

68. The method of claim 67, wherein the transcription factor is a maf family protein.

69. The method of claim 68, wherein the maf family protein is c-Maf.

70. The method of claim 68, wherein the maf family protein is a small maf protein.

71. The method of claim 67, wherein the transcription factor interacts with an NF-AT family protein.

72. The method of claim 71, wherein the transcription factor is NIP45.

73. The method of claim 67, wherein the indicator composition is a lymphoid cell.

74. The method of claim 73, wherein the lymphoid cell is a Th2 cell.

75. The method of claim 67, wherein the indicator composition is a yeast cell.

76. The method of any of claims 67-75, wherein the indicator composition comprises an indicator cell, wherein said indicator cell comprises: (i) the transcription factor and (ii) a reporter gene responsive to the transcription factor.

77. The method of claim 76, wherein said indicator cell contains:

i) a recombinant expression vector encoding the transcription factor; and

ii) a vector comprising regulatory sequences of a Th2-associated cytokine gene operatively linked a reporter gene; and

said method comprises:

a) contacting the indicator cell with a test compound;

b) determining the level of expression of the reporter gene in the indicator cell in the presence of the test compound; and

c) comparing the level of expression of the reporter gene in the indicator cell in the presence of the test compound with the level of expression of the reporter gene in the indicator cell in the absence of the test compound to thereby identify a compound that modulates the activity of the transcription factor.

78. The method of any of claims 67-72, wherein the indicator composition comprises a preparation of: (i) the transcription factor and (ii) a DNA molecule to which the transcription factor binds, and

said method comprises:

a) contacting the indicator composition with a test compound;

b) determining the degree of interaction of the transcription factor and the DNA molecule in the presence of the test compound; and

c) comparing the degree of interaction of the transcription factor and the DNA molecule in the presence of the test compound with the degree of interaction of the transcription factor and the DNA molecule in the absence of the test compound to thereby identify a compound that modulates the activity of the transcription factor.

79. The method of claim 78, wherein the transcription factor is a maf family protein and the DNA molecule comprises a maf response element (MARE).

80. The method of any of claims 67-75, which identifies a protein from Th2 cells that interacts with the transcription factor, wherein:

the indicator composition is an indicator cell, which indicator cell comprises:

i) a reporter gene operably linked to a transcriptional regulatory sequence; and

ii) a first chimeric gene which encodes a first fusion protein, said first fusion protein including the transcription factor that cooperates with an NFAT family protein to regulate expression of a Th2-associated cytokine gene;

the test compound comprises a library of second chimeric genes, which library encodes second fusion proteins, the second fusion proteins including proteins derived from Th2 cells;

expression of the reporter gene being sensitive to interactions between the first fusion protein, the second fusion protein and the transcriptional regulatory sequence; and

wherein the effect of the test compound on the transcription factor in the indicator composition is determined by determining the level of expression of the reporter gene in the indicator cell to thereby identify a test compound comprising a protein from Th2 cells that interacts with the transcription factor.

81. A method for identifying a compound that modulates an interaction between NIP45 and an NF-AT family protein, comprising:

a) combining:

(i) NIP45, or an NF-AT-interacting portion thereof; and

(ii) an NF-AT family protein, or a NIP45-interacting portion thereof;

in the presence and absence of a test compound;

b) determining the degree of interaction between (i) and (ii) in the presence and absence of the test compound; and

c) identifying a compound that modulates an interaction between NIP45 and an NF-AT family protein.

82. The method of claim 81, wherein the NIP45-interacting portion of the NF-AT family protein comprises the Rel Homology Domain of the NF-AT family protein.

83. The method of claim 81, wherein the degree of interaction between (i) and (ii) is determined by labeling (i) or (ii) with a detectable substance, isolating non-labeled (i) or (ii) and quantitating the amount of labeled (i) or (ii) that has become associated with non-labeled (i) or (ii).

84. The method of any of claims 67-75 or 81-83, further comprising determining the effect of the compound on an immune response to thereby identify a compound that modulates an immune response.

85. The method of claim 84, wherein the effect of the compound on an immune response is determined by determining the effect of the compound on expression of a Th2-associated cytokine gene.

86. The method of claim 85, wherein the Th2-associated cytokine gene is an interleukin-4 gene.

87. The method of claim 84, wherein the effect of the compound of interest on an immune response is determined by determining the effect of the compound on development of T helper type 1 (Th1) or T helper type 2 (Th2) cells.

FIG.1A

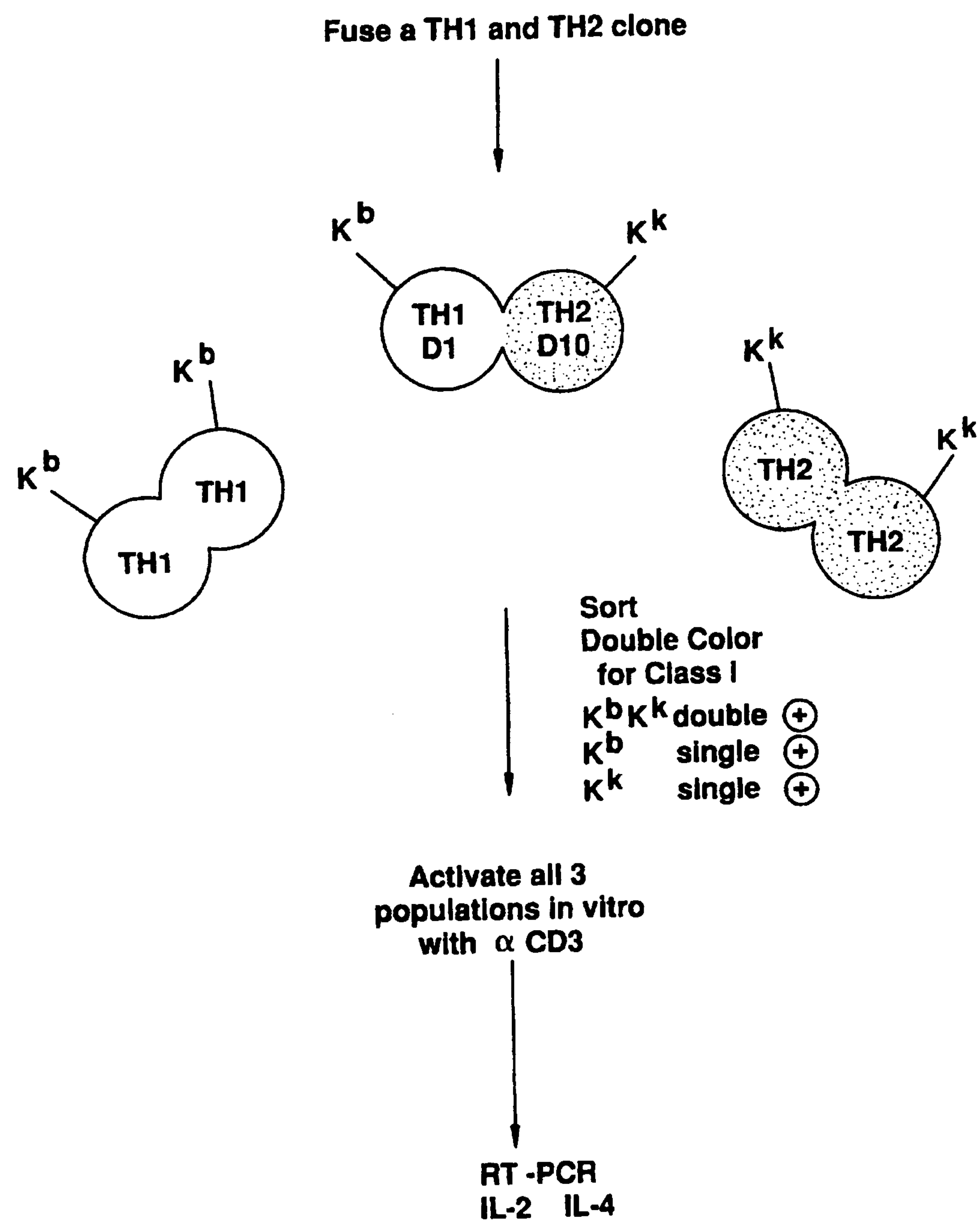
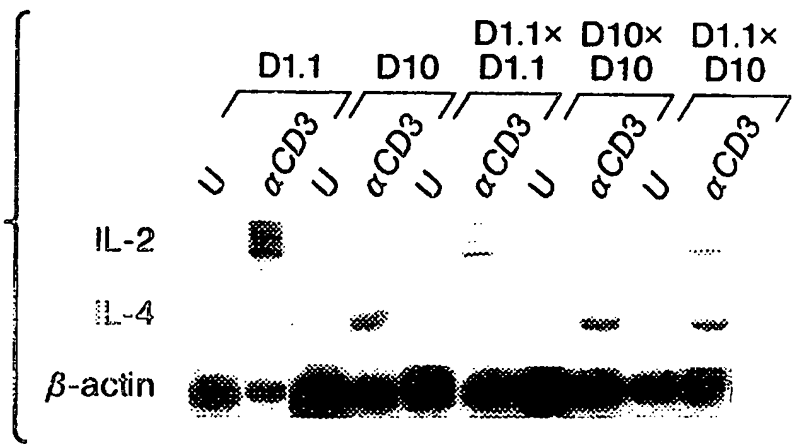

Fuse a TH1 and TH2 clone
K^b
K^k
TH1 D1
TH2 D10
K^b
K^b
TH1
TH1
K^k
K^k
TH2
TH2
Sort
Double Color
for Class I
K^bK^k double ⊕
K^b single ⊕
K^k single ⊕
Activate all 3 populations in vitro with α CD3
RT -PCR
IL-2 IL-4

FIG.1B


D1.1
D10
D1.1×
D1.1
D10×
D10
D1.1×
D10
U
αCD3
U
αCD3
U
αCD3
U
αCD3
U
αCD3
IL-2
IL-4
β-actin

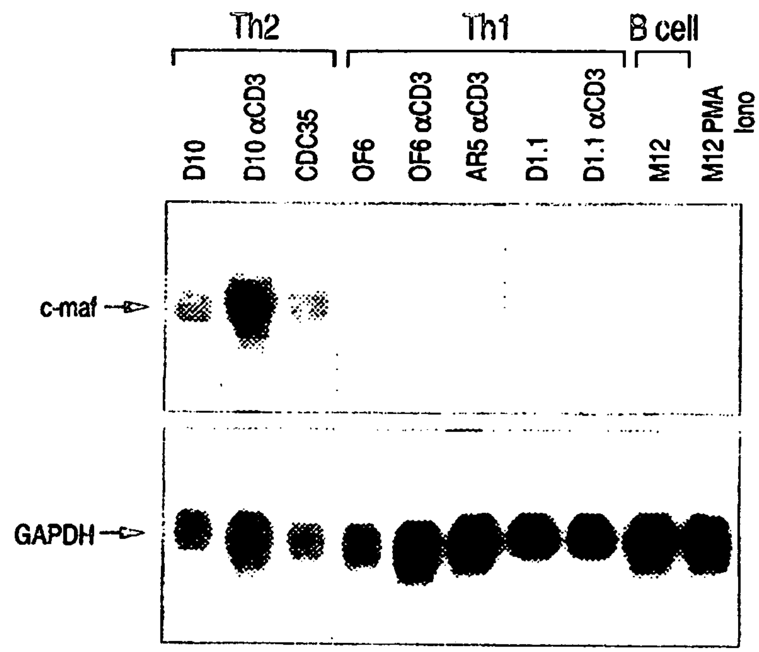
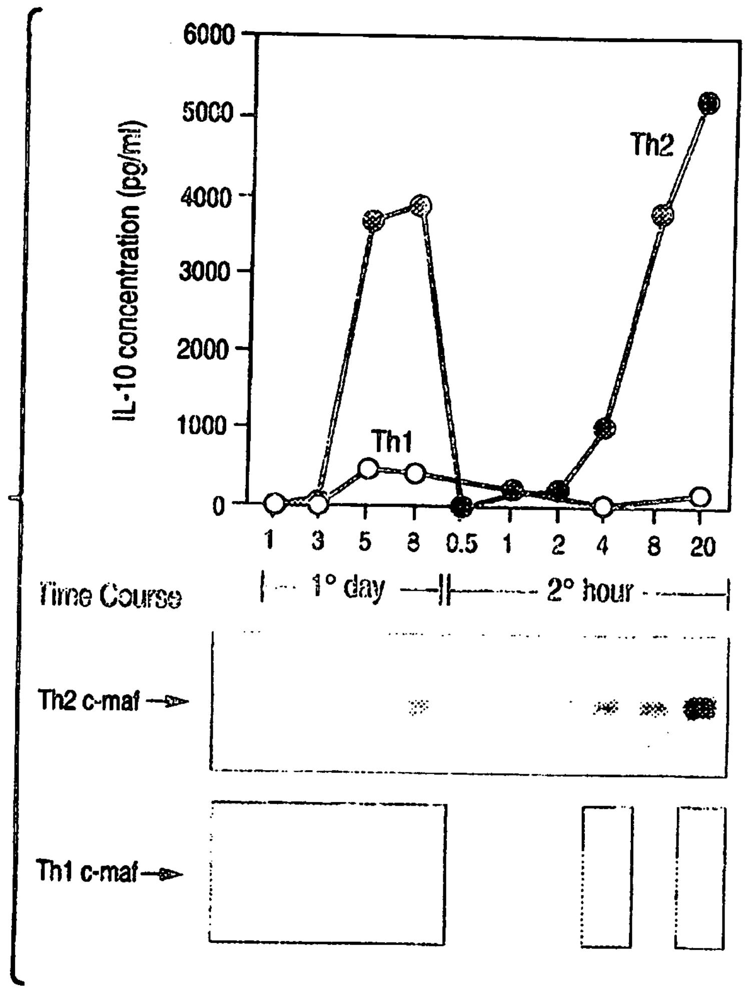

Th2
Th1
B cell
D10
D10 αCD3
CDC35
OF6
OF6 αCD3
AR5 αCD3
D1.1
D1.1 αCD3
M12
M12 PMA Iono
c-maf
GAPDH

FIG.2A

FIG.2B


6000
5000
4000
3000
2000
1000
0
IL-10 concentration (pg/ml)
Th2
Th1
1
3
5
8
0.5
1
2
4
8
20
Time Course
1° day
2° hour
Th2 c-maf
Th1 c-maf

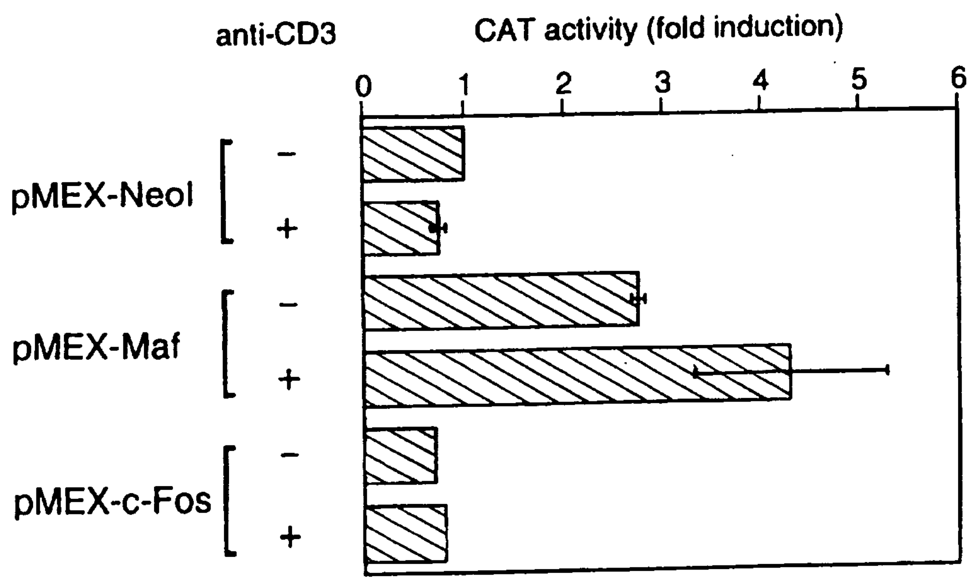
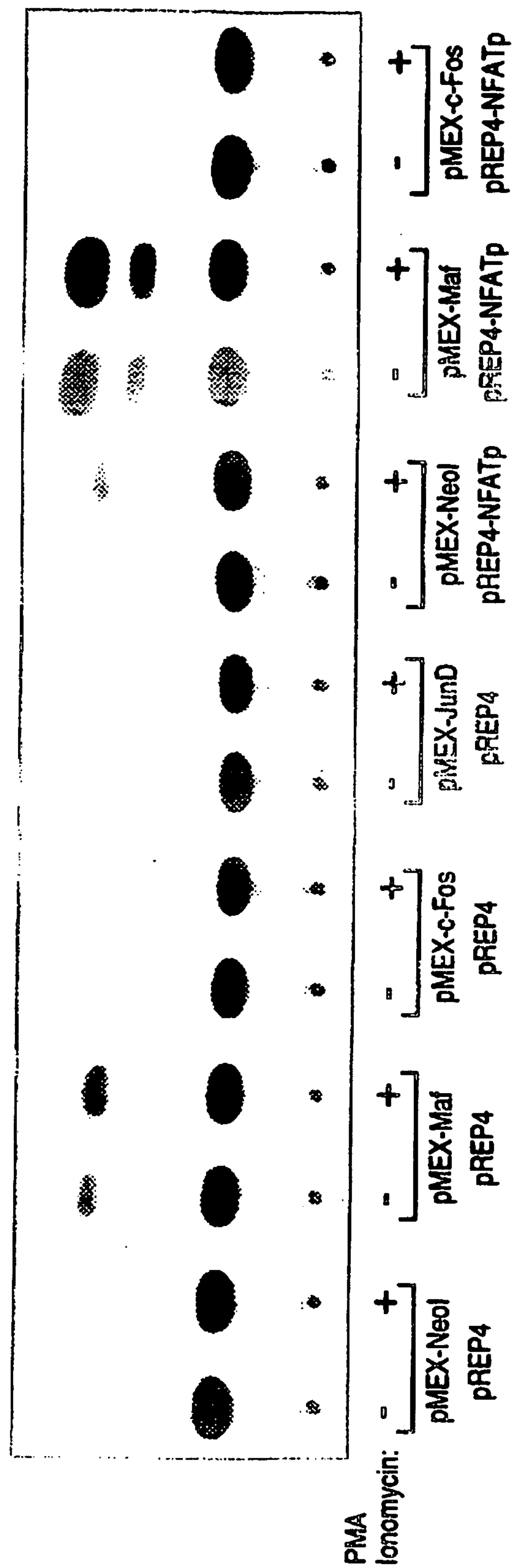

anti-CD3
CAT activity (fold induction)
0
1
2
3
4
5
6
pMEX-NeoI
-
+
pMEX-Maf
-
+
pMEX-c-Fos
-
+

FIG.3A

FIG.3B


PMA
Ionomycin:
- +
pMEX-Neol
pREP4
- +
pMEX-Maf
pREP4
- +
pMEX-c-Fos
pREP4
- +
pMEX-JunD
pREP4
- +
pMEX-Neol
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pMEX-c-Fos
pREP4-NFATp

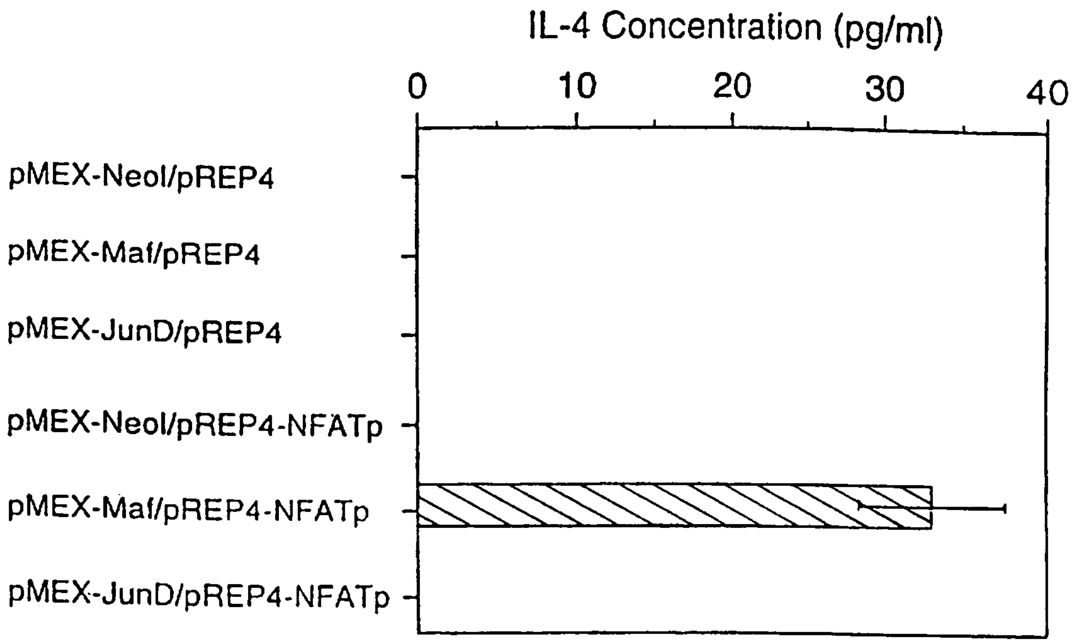
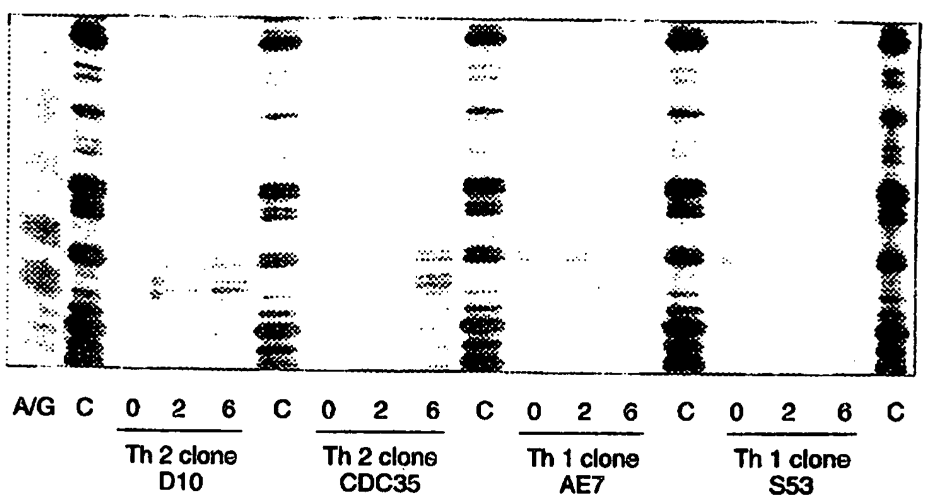
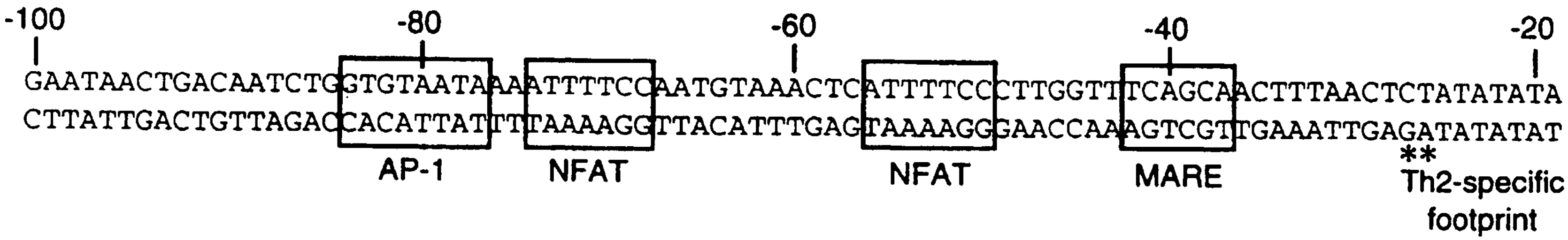

IL-4 Concentration (pg/ml)
0
10
20
30
40
pMEX-Neol/pREP4
pMEX-Maf/pREP4
pMEX-JunD/pREP4
pMEX-Neol/pREP4-NFATp
pMEX-Maf/pREP4-NFATp
pMEX-JunD/pREP4-NFATp

FIG.4

FIG.5A


A/G C 0 2 6 C 0 2 6 C 0 2 6 C 0 2 6 C
Th 2 clone D10
Th 2 clone CDC35
Th 1 clone AE7
Th 1 clone S53



WT -27 to -59	CTCATTTTCCCTTGGTTTCAGCAACTTTAACTC
mut 2	CTCATTTTCCCTTGGTTTCAGCAAC**gggc**ACTC
mut 3	CTCATTTTCCCTTGGTTTCAG**acca**TTTAACTC
mut 4	CTCATTTTCCCTTGGTT**gact**CAACTTTAACTC
mut 5	CTCATTTTCCCTT**ttgg**TCAGCAACTTTAACTC
mut 6	CTCATTTTC**aagg**GGTTTCAGCAACTTTAACTC
mut 7	CTCAT**ggga**CCTTGGTTTCAGCAACTTTAACTC
mut 8	C**gacg**TTTCCCTTGGTTTCAGCAACTTTAACTC

FIG.5B

FIG.6

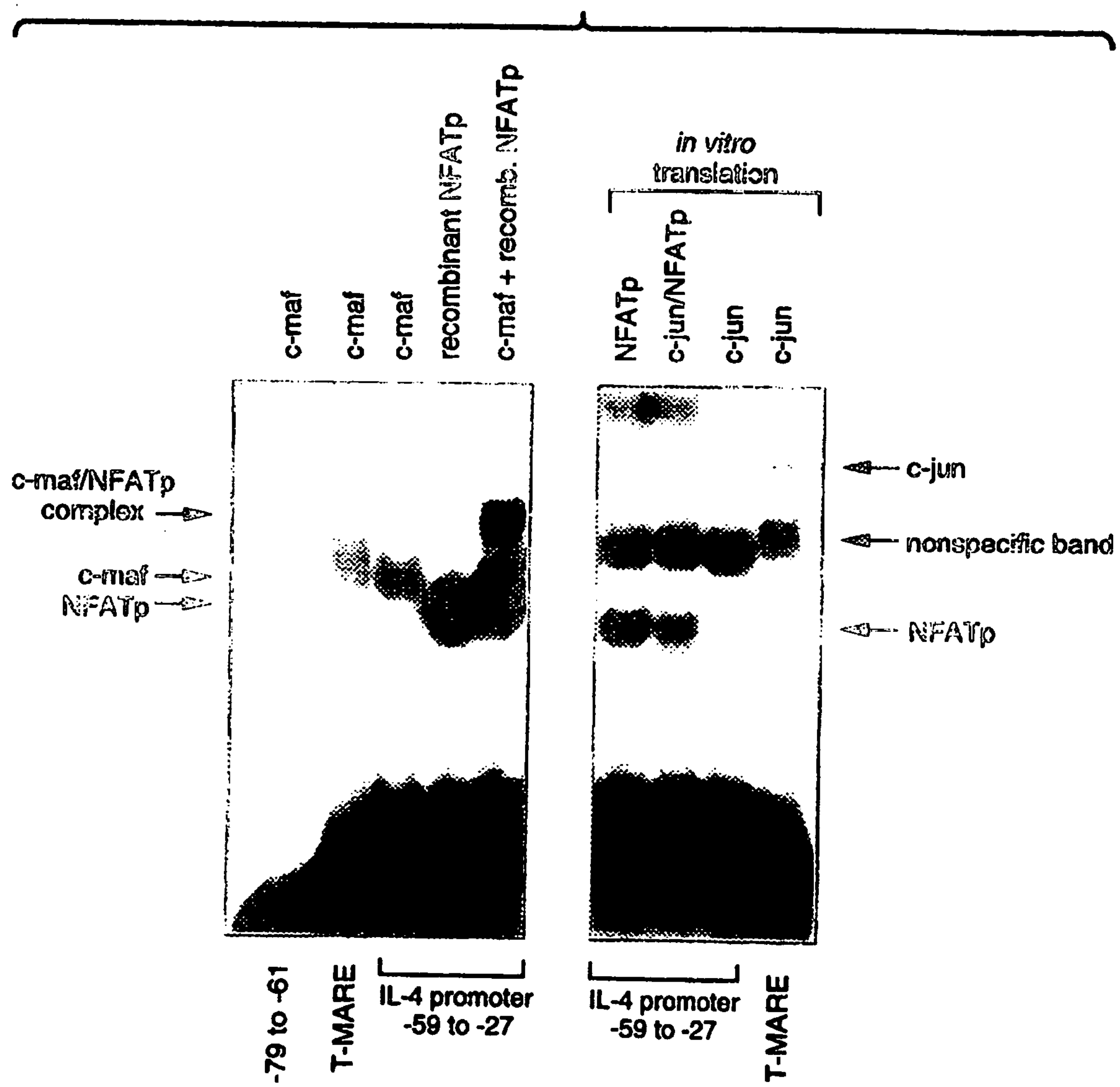

c-maf
c-maf
c-maf
recombinant NFATp
c-maf + recomb. NFATp
in vitro translation
NFATp
c-jun/NFATp
c-jun
c-jun
c-maf/NFATp complex
c-maf
NFATp
c-jun
nonspecific band
NFATp
-79 to -61
T-MARE
IL-4 promoter -59 to -27
IL-4 promoter -59 to -27
T-MARE

FIG. 7A

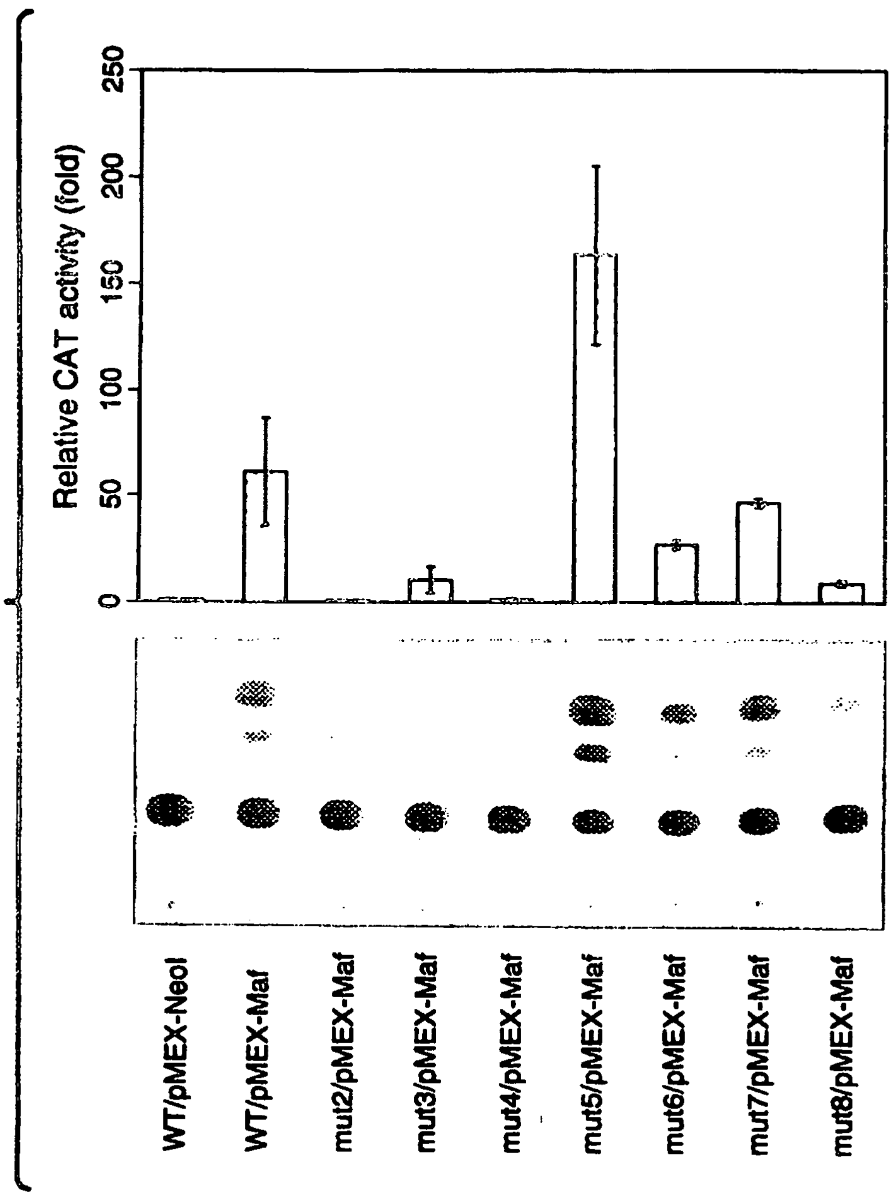
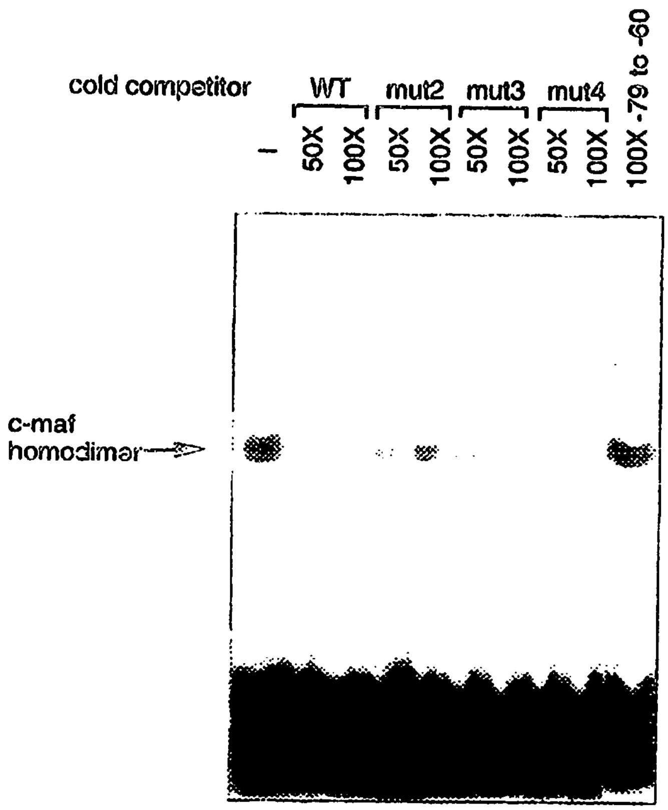

Relative CAT activity (fold)
0
50
100
150
200
250
WT/pMEX-Neol
WT/pMEX-Maf
mut2/pMEX-Maf
mut3/pMEX-Maf
mut4/pMEX-Maf
mut5/pMEX-Maf
mut6/pMEX-Maf
mut7/pMEX-Maf
mut8/pMEX-Maf

FIG.7B



IL-4 promoter -59 to -27 probe

FIG.8

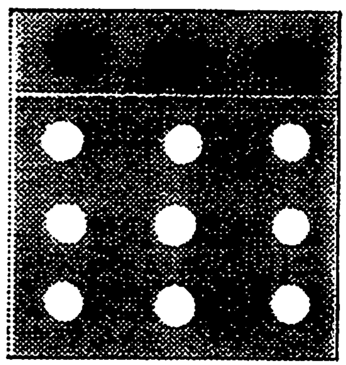
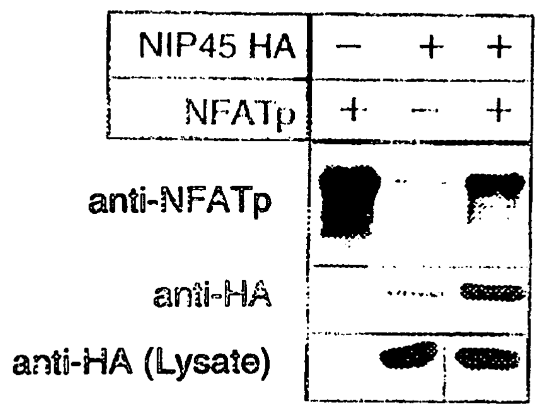


FIG.9



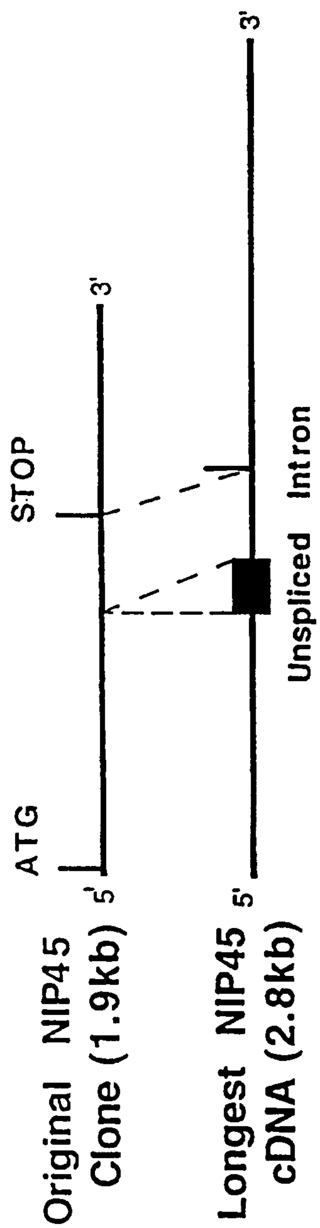

Original NIP45
Clone (1.9kb)
Longest NIP45
cDNA (2.8kb)
ATG
STOP
5'
3'
5'
3'
Unspliced Intron

FIG.10

ACAGTGTGGGAGATGGCGGAACCACTGAGGGGACGTGGTCCGAGGTCC 48
TGTCACACCCTCTACCGCCTTGGTGACTCCCCTGCACCAGGCTCCAGG
M A E P L R G R G P R S **12**

CGCGGTGGCCGAGGCGCTCGGAGAGCCCGAGGCGCCCGTGGCCGGTGT 96
GCGCCACCGGCTCCGCGAGCCTCTCGGGCTCCGCGGGCACCGGCCACA
R G G R G A R R A R G A R G R C **28**

CCTCGCGCCCGGCAGTCTCCGGCTAGGCTCATTCCAGACACCGTGCTT 144
GGAGCGCGGGCCGTCAGAGGCCGATCCGAGTAAGGTCTGTGGCACGAA
P R A R Q S P A R L I P D T V L **44**

GTGGACTTGGTCAGTGACAGCGACGAAGAGGTCTTGGAAGTCGCAGAC 192
CACCTGAACCAGTCACTGTCGCTGCTTCTCCAGAACCTTCAGCGTCTG
V D L V S D S D E E V L E V A D **60**

CCAGTAGAGGTGCCGGTCGCCCGCCTCCCCGCGCCGGCTAAACCTGAG 240
GGTCATCTCCACGGCCAGCGGGCGGAGGGGCGCGGCCGATTTGGACTC
P V E V P V A R L P A P A K P E **76**

CAGGACAGCGACAGTGACAGTGAAGGGGCGGCCGAGGGGCCTGCGGGA 288
GTCCTGTCGCTGTCACTGTCACTTCCCCGCCGGCTCCCCGGACGCCCT
Q D S D S D S E G A A E G P A G **92**

GCCCCGCGTACATTGGTGCGACGGCGGCGGCGGCGGCTGCTGGATCCC 336
CGGGGCGCATGTAACCACGCTGCCGCCGCCGCCGCCGACGACCTAGGG
A P R T L V R R R R R R L L D P **108**

GGAGAGGCGCCGGTGGTCCCAGTGTACTCCGGGAAGGTACAGAGCAGC 384
CCTCTCCGCGGCCACCAGGGTCACATGAGGCCCTTCCATGTCTCGTCG
G E A P V V P V Y S G K V Q S S **124**

CTCAACCTCATTCCAGATAATTCATCCCTCTTGAAACTGTGCCCTTCA 432
GAGTTGGAGTAAGGTCTATTAAGTAGGGAGAACTTTGACACGGGAAGT
L N L I P D N S S L L K L C P S **140**

GAGCCTGAAGATGAGGCAGATCTGACAAATTCTGGCAGTTCTCCCTCT 480
CTCGGACTTCTACTCCGTCTAGACTGTTTAAGACCGTCAAGAGGGAGA
E P E D E A D L T N S G S S P S **156**

FIGURE 11A

```
GAGGATGATGCCCTGCCTTCAGGTTCTCCCTGGAGAAAGAAGCTCAGA   528
CTCCTACTACGGGACGGAAGTCCAAGAGGGACCTCTTTCTTCGAGTCT
 E  D  D  A  L  P  S  G  S  P  W  R  K  K  L  R    172

AAGAAGTGTGAGAAAGAAGAAAAGAAAATGGAAGAGTTTCCGGACCAG   576
TTCTTCACACTCTTTCTTCTTTTCTTTTACCTTCTCAAAGGCCTGGTC
 K  K  C  E  K  E  E  K  K  M  E  E  F  P  D  Q    188

GACATCTCTCCTTTGCCCCAACCTTCGTCAAGGAACAAAAGCAGAAAG   624
CTGTAGAGAGGAAACGGGGTTGGAAGCAGTTCCTTGTTTTCGTCTTTC
 D  I  S  P  L  P  Q  P  S  S  R  N  K  S  R  K    204

CATACGGAGGCGCTCCAGAAGCTAAGGGAAGTGAACAAGCGTCTCCAA   672
GTATGCCTCCGCGAGGTCTTCGATTCCCTTCACTTGTTCGCAGAGGTT
 H  T  E  A  L  Q  K  L  R  E  V  N  K  R  L  Q    220

GATCTCCGCTCCTGCCTGAGCCCCAAGCAGCACCAGAGTCCAGCCCTT   720
CTAGAGGCGAGGACGGACTCGGGGTTCGTCGTGGTCTCAGGTCGGGAA
 D  L  R  S  C  L  S  P  K  Q  H  Q  S  P  A  L    236

CAGAGCACAGATGATGAGGTGGTCCTAGTGGAAGGGCCTGTCTTGCCA   768
GTCTCGTGTCTACTACTCCACCAGGATCACCTTCCCGGACAGAACGGT
 Q  S  T  D  D  E  V  V  L  V  E  G  P  V  L  P    252

CAGAGCTCTCGACTCTTTACACTCAAGATCCGGTGCCGGGCTGACCTA   816
GTCTCGAGAGCTGAGAAATGTGAGTTCTAGGCCACGGCCCGACTGGAT
 Q  S  S  R  L  F  T  L  K  I  R  C  R  A  D  L    268

GTGAGACTGCCTGTCAGGATGTCGGAGCCCCTTCAGAATGTGGTGGAT   864
CACTCTGACGGACAGTCCTACAGCCTCGGGGAAGTCTTACACCACCTA
 V  R  L  P  V  R  M  S  E  P  L  Q  N  V  V  D    284

CACATGGCCAATCATCTTGGGGTGTCTCCAAACAGGATTCTTTTGCTT   912
GTGTACCGGTTAGTAGAACCCCACAGAGGTTTGTCCTAAGAAAACGAA
 H  M  A  N  H  L  G  V  S  P  N  R  I  L  L  L    300

TTTGGAGAGAGTGAACTGTCTCCTACTGCCACCCCTAGTACCCTAAAG   960
AAACCTCTCTCACTTGACAGAGGATGACGGTGGGGATCATGGGATTTC
 F  G  E  S  E  L  S  P  T  A  T  P  S  T  L  K    316
```

FIGURE 11B

```
CTTGGAGTGGCTGACATCATTGATTGTGTGGTGCTAGCAAGCTCTTCA    1008
GAACCTCACCGACTGTAGTAACTAACACACCACGATCGTTCGAGAAGT
 L  G  V  A  D  I  I  D  C  V  V  L  A  S  S  S     312

GAGGCCACAGAGACATCCCAGGAGCTCCGGCTCCGGGTGCAGGGGAAG    1056
CTCCGGTGTCTCTGTAGGGTCCTCGAGGCCGAGGCCCACGTCCCCTTC
 E  A  T  E  T  S  Q  E  L  R  L  R  V  Q  G  K     348

GAGAAACACCAGATGTTGGAGATCTCACTGTCTCCTGATTCTCCTCTT    1104
CTCTTTGTGGTCTACAACCTCTAGAGTGACAGAGGACTAAGAGGAGAA
 E  K  H  Q  M  L  E  I  S  L  S  P  D  S  P  L     364

AAGGTTCTCATGTCACACTATGAGGAAGCCATGGGACTCTCTGGACAC    1152
TTCCAAGAGTACAGTGTGATACTCCTTCGGTACCCTGAGAGACCTGTG
 K  V  L  M  S  H  Y  E  E  A  M  G  L  S  G  H     380

AAGCTCTCCTTCTTCTTTGATGGGACAAAGCTTTCAGGCAAGGAGCTG    1200
TTCGAGAGGAAGAAGAAACTACCCTGTTTCGAAAGTCCGTTCCTCGAC
 K  L  S  F  F  F  D  G  T  K  L  S  G  K  E  L     396

CCAGCTGATCTGGGCCTGGAATCCGGAGATCTCATCGAAGTCTGGGGC    1248
GGTCGACTAGACCCGGACCTTAGGCCTCTAGAGTAGCTTCAGACCCCG
 P  A  D  L  G  L  E  S  G  D  L  I  E  V  W  G     412

TGAAGCTCTCACCCTGTTCGGACGCAAAGCCAAGACATGGAGACAATA    1296
ACTTCGAGAGTGGGACAAGCCTGCGTTTCGGTTCTGTACCTCTGTTAT

GCTCCCAATTTTATTATTGTGATTTTTCGCCCCATAAGGGCTAACAGA    1344
CGAGGGTTAAAATAATAACACTAAAAAGCGGGGTATTCCCGATTGTCT

AACTGAATTAGAACTTGTTTACTTATTTATTTCTGGTGCTGGGGATTG    1392
TTGACTTAATCTTGAACAAATGAATAAATAAAGACCACGACCCCTAAC

AACCCCAGACTATGCACATGCTAAGGATGTATGAAGTGGAGGCAAAAC    1440
TTGGGGTCTGATACGTGTACGATTCCTACATACTTCACCTCCGTTTTG

CAAGGCATTACCTTTAGCCAGCCTCTAGTAGACTGTAGTGTCAAGCAA    1488
GTTCCGTAATGGAAATCGGTCGGAGATCATCTGACATCACAGTTCGTT
```

FIGURE 11C

GTGGCTACTTGGTAGTTGTGTGGCTCTGTGTATGTTTGTGCTGTATTT 1536
CACCGATGAACCATCAACACACCGAGACACATACAAACACGACATAAA

GGCAGCCCCTGGGGCACATAGAAGGGACCTTGGCTTCCCTACCATTTC 1584
CCGTCGGGGACCCCGTGTATCTTCCCTGGAACCGAAGGGATGGTAAAG

ACGTTCGCTGGTGCCCTTTCCTTCATCAGATGACTTCTGTGAAGCTGC 1632
TGCAAGCGACCACGGGAAAGGAAGTAGTCTACTGAAGACACTTCGACG

CTATGTTGAGTGTGTTGAACTAAATGAGCTCTGCTTTGGGTGTCCAGG 1680
GATACAACTCACACAACTTGATTTACTCGAGACGAAACCCACAGGTCC

CCTGGGGTTTGTGCCGCAGTTGGAGCCAGCAGTGACTTCACTCTGACT 1728
GGACCCCAAACACGGCGTCAACCTCGGTCGTCACTGAAGTGAGACTGA

TGGGACTGAGAATGCATTTCCTGGTGGAGACACTCGGGTGCAGAAATA 1776
ACCCTGACTCTTACGTAAAGGACCACCTCTGTGAGCCCACGTCTTTAT

TAACAGAAGGTGACATACATGCTGAAGCTGAGGACTAGGTCGAAAGTT 1824
ATTGTCTTCCACTGTATGTACGACTTCGACTCCTGATCCAGCTTTCAA

AACGACGTTGCATTTTCAGCCTTGGGTATCCTCTCTGCCTGCCAGGAC 1872
TTGCTGCAACGTAAAAGTCGGAACCCATAGGAGAGACGGACGGTCCTG

TCTAGCCAGTGTCTGGTACACACTTCTTGGCATGGACACCTAGGTCGA 1920
AGATCGGTCACAGACCATGTGTGAAGAACCGTACCTGTGGATCCAGCT

CGCGGGCGCGATTCGGCCGACTCGAG 1946
GCGCCCGCGCTAAGCCGGCTGAGCTC

FIGURE 11D

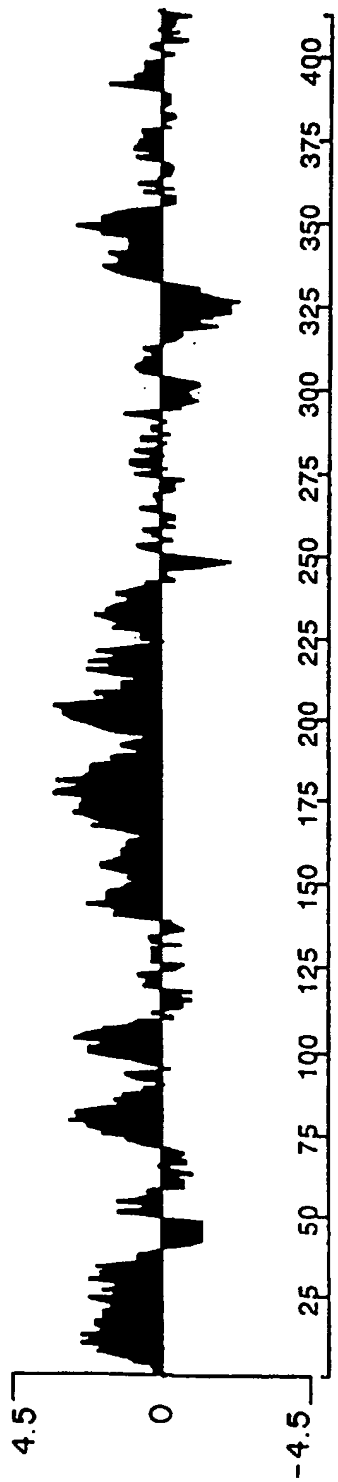

4.5
0
-4.5
25
50
75
100
125
150
175
200
225
250
275
300
325
350
375
400

FIG.12

FIG.13

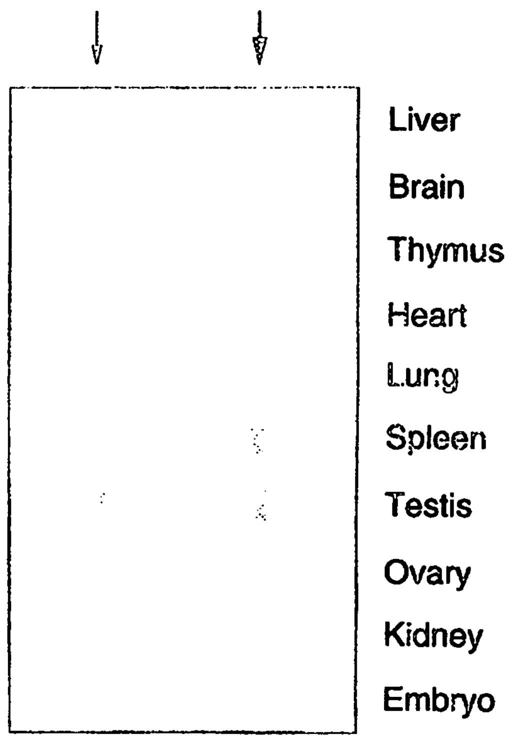

Liver
Brain
Thymus
Heart
Lung
Spleen
Testis
Ovary
Kidney
Embryo

FIG.14A

FIG.14B

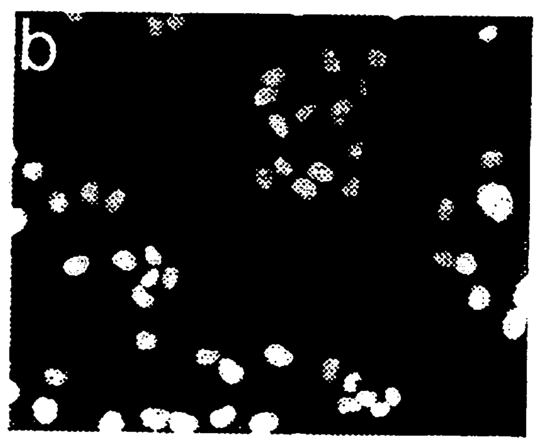


FIG.14C

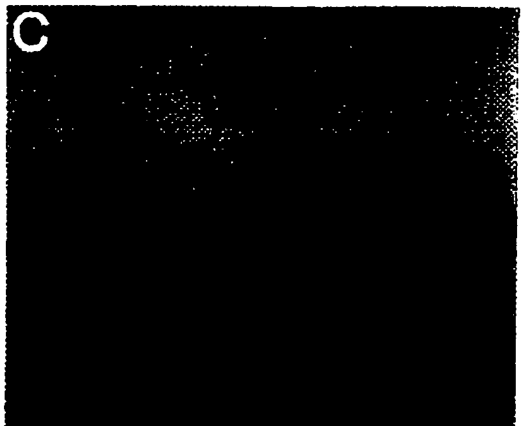
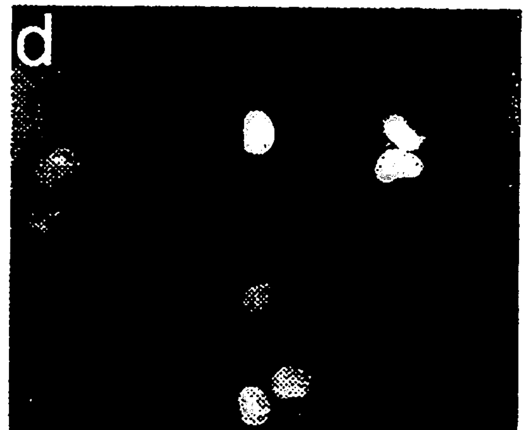


FIG.14D



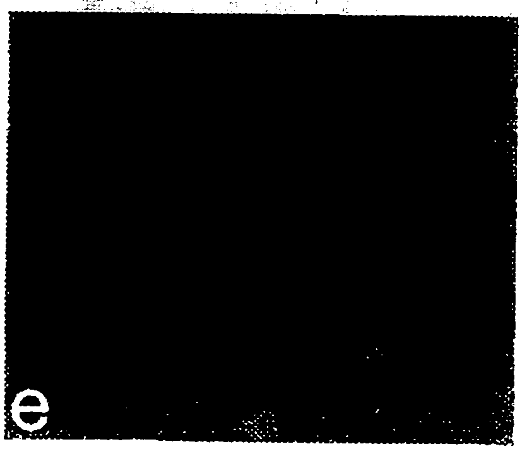


FIG.14E

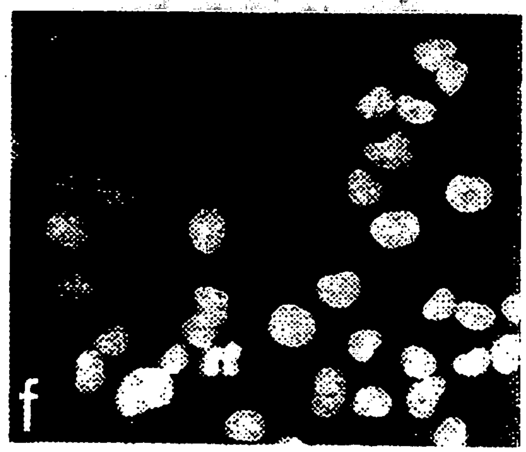


FIG.14F

FIG.15

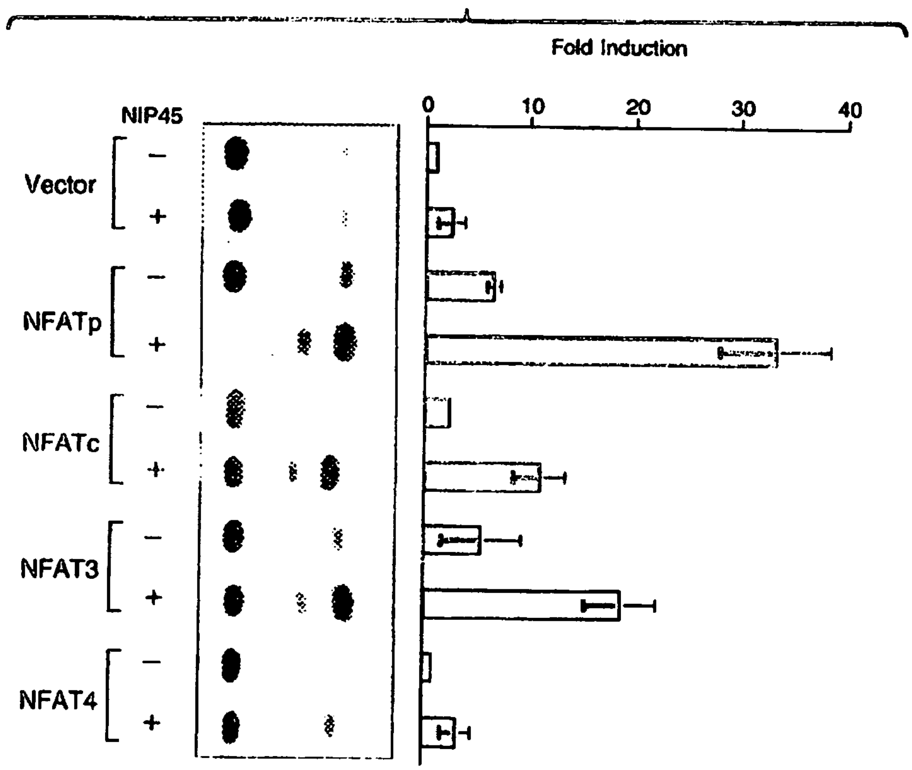
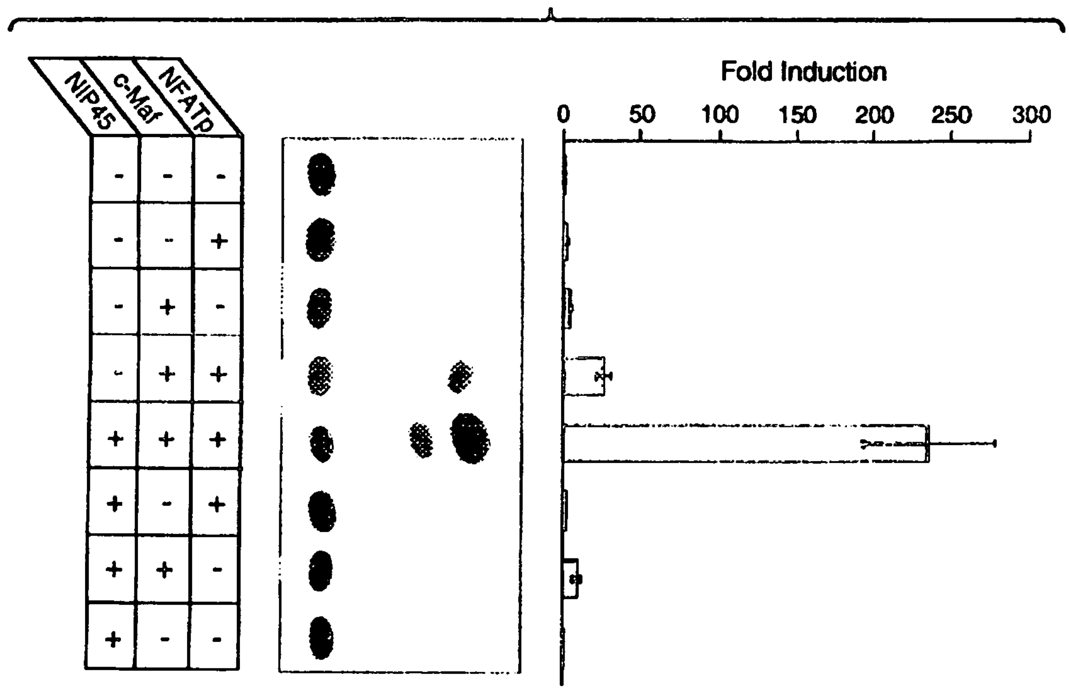

Fold Induction
0
10
20
30
40
NIP45
Vector
−
+
NFATp
−
+
NFATc
−
+
NFAT3
−
+
NFAT4
−
+

FIG.16



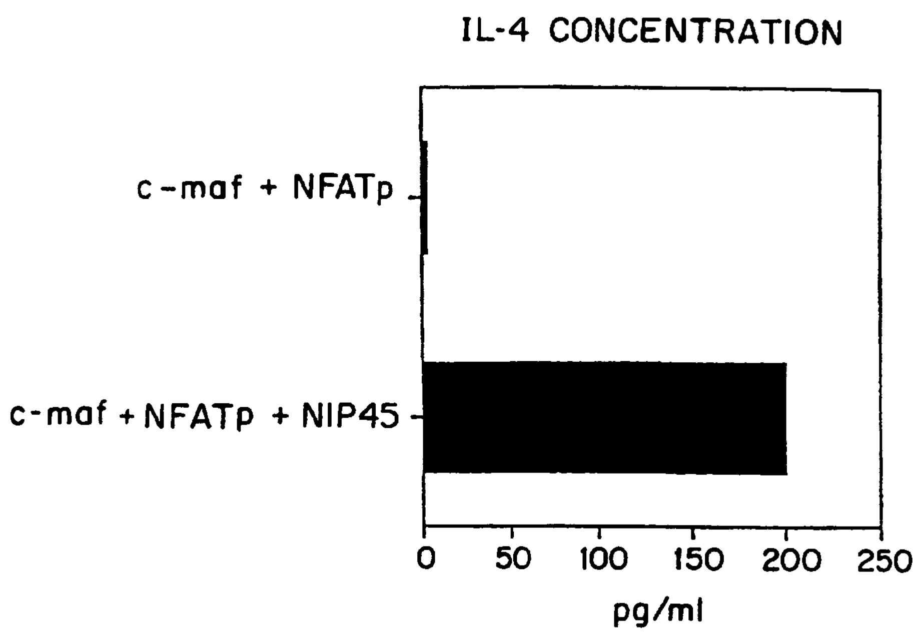

IL-4 CONCENTRATION
c-maf + NFATp
c-maf + NFATp + NIP45
0
50
100
150
200
250
pg/ml

FIG. 17

FIG. 18

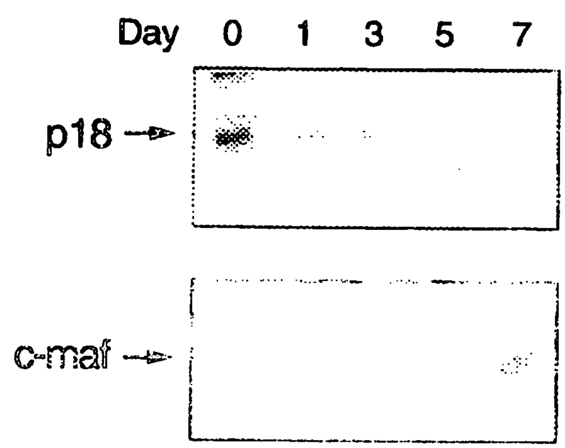
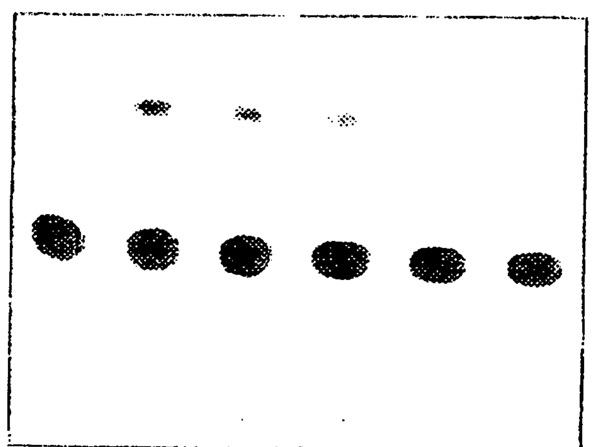


FIG.19



c-maf (µg)	–	5	5	5	5	–
p18 (µg)	–	–	2.5	5	10	10

FIG.20

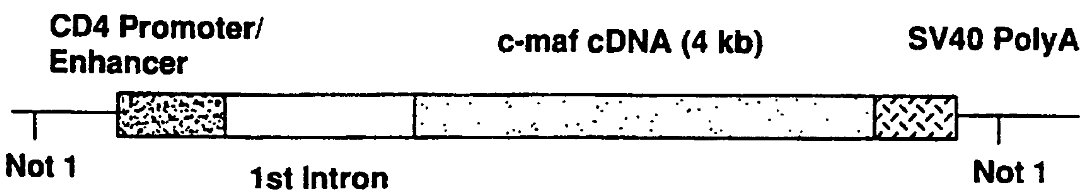

CD4 Promoter/
Enhancer
c-maf cDNA (4 kb)
SV40 PolyA
Not 1
1st Intron
Not 1

FIG.21

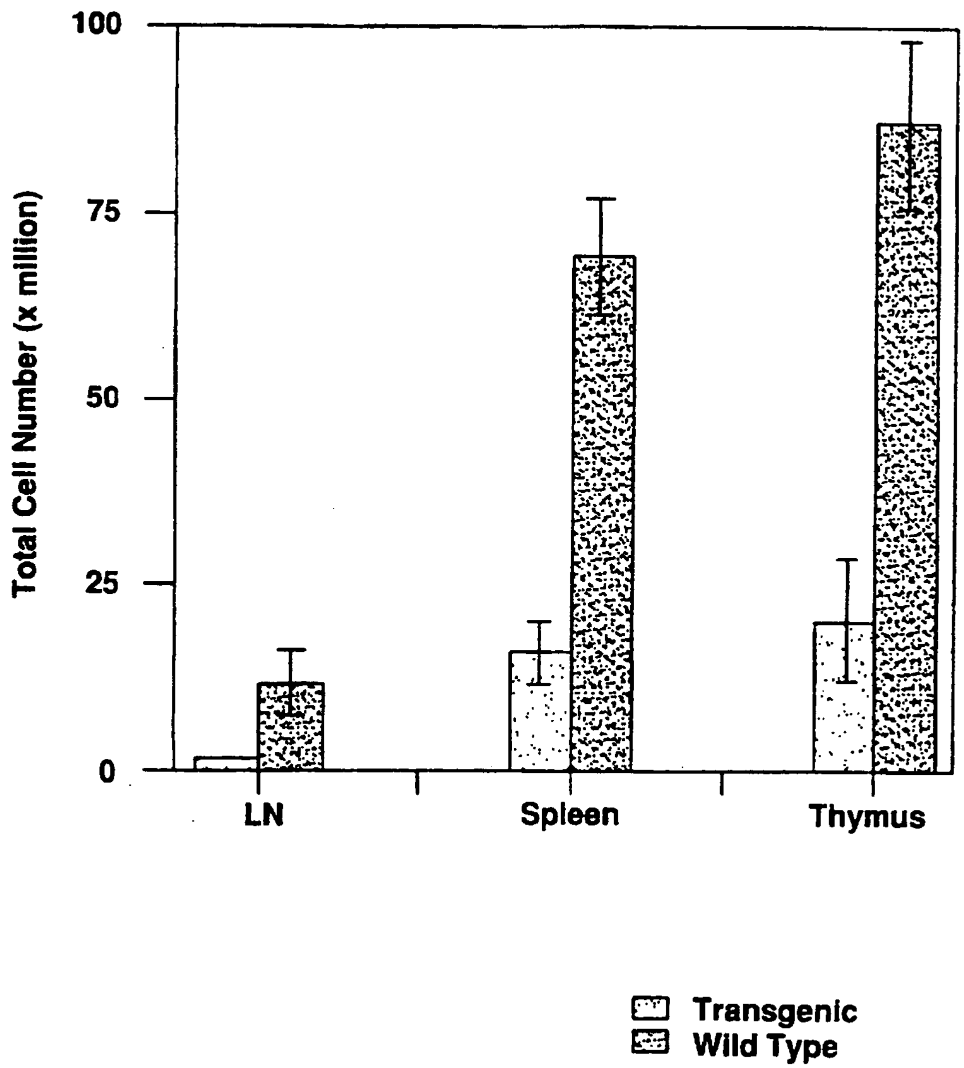
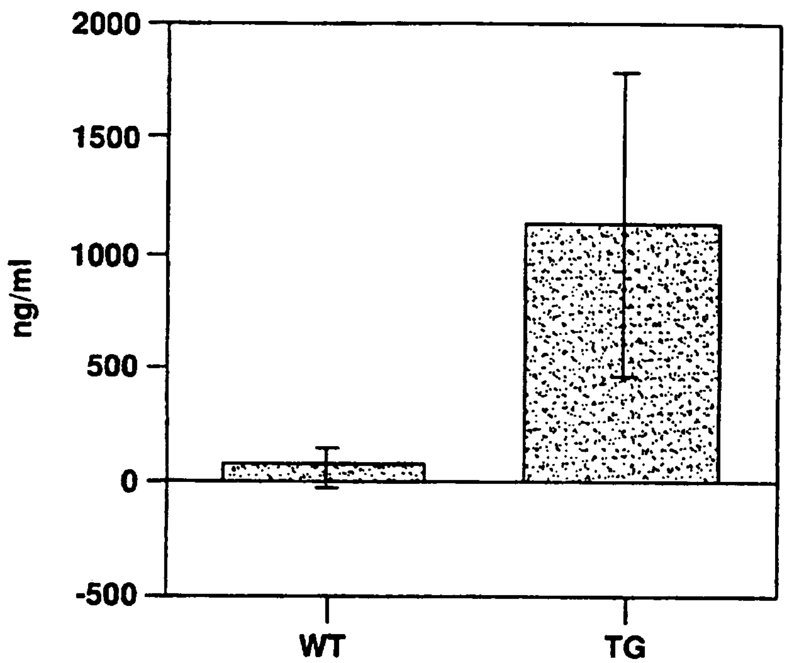

100
75
50
25
0
Total Cell Number (x million)
LN
Spleen
Thymus
Transgenic
Wild Type

FIG.22



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08/755,592	25 November 1996 (25.11.96)	US

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(63) Related by Continuation

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Filed on	23 April 1996 (23.04.96)
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US	08/755,592 (CIP)
Filed on	25 November 1996 (25.11.96)

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(74) Agents: KARA, Catherine, J. et al.; Lahive & Cockfield, L.L.P., 28 State Street, Boston, MA 02109 (US).

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(54) Title: METHODS AND COMPOSITIONS FOR REGULATING T CELL SUBSETS BY MODULATING TRANSCRIPTION FACTOR ACTIVITY

(57) Abstract

Methods for modulating production of a T helper type 2 (Th2)-associated cytokine, in particular interleukin-4, by modulating the activity of one or more transcription factors that cooperate with NF-AT family proteins to regulate expression of a Th2-associated cytokine gene are disclosed. In one embodiment, the activity of a maf family protein (e.g., c-Maf or a small maf protein, such as p18) is modulated. In another embodiment, the activity of a protein that interacts with an NF-AT family protein (e.g., NIP45) is modulated. Combination methods, for example wherein the activities of a maf family protein and an NF-AT protein are modulated or the activities of a maf protein and an NF-AT-interacting protein are modulated, are also encompassed by the invention. Methods for modulating development of T helper type 1 (Th1) or T helper type 2 (Th2) subsets in a subject using agents that modulate transcription factor activity are also disclosed. NIP45 compositions, including isolated nucleic acid molecules encoding NIP45, antisense nucleic acid molecules, recombinant expression vectors containing a NIP45 nucleic acid molecule, host cells into which such expression vectors have been introduced and non-human transgenic animals carrying a NIP45 transgene, are also provided by the invention. The invention further provides isolated NIP45 proteins and peptides, NIP45 fusion proteins and anti-NIP45 antibodies. Methods of using the NIP45 compositions of the invention are also disclosed.

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INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 97/06708

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C12N5/10 C07K16/18 C12N15/62 A01K67/027
G01N33/68 C12N15/63 C07K14/47

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A01N G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HODGE M. R. ET AL: "Hyperproliferation and dysregulation of IL-4 expression in NF-ATp-deficient mice" IMMUNITY, vol. 4, April 1996, pages 397-405, XP002045449 see the whole document ---	1-21, 30-64, 67-87
Y	ROONEY J W ET AL: "Coordinate and cooperative roles for NF -AT and AP-1 in the regulation of the murine IL - 4 gene." IMMUNITY, vol. 2, May 1995, pages 473-483, XP002045450 cited in the application see the whole document ---	1-21, 30-64, 67-87
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[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 February 1998	03-03-1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hix, R

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HODGE M R ET AL: "The proximal promoter of the IL - 4 gene is composed of multiple essential regulatory sites that bind at least two distinct factors." JOURNAL OF IMMUNOLOGY 154 (12). 1995. 6397-6405. ISSN: 0022-1767, vol. 154, no. 12, 1995, pages 6397-6405, XP002045451 see the whole document ---	1-21, 30-64, 67-87
Y	ROONEY J W ET AL: "A common factor regulates both Th1- and Th2 -specific cytokine gene expression." EMBO (EUROPEAN MOLECULAR BIOLOGY ORGANIZATION) JOURNAL 13 (3). 1994. 625-633. ISSN: 0261-4189, XP002045452 see the whole document ---	1-21, 30-64, 67-87
Y	CASOLARO V ET AL: "Inhibition of NF -AT-dependent transcription by NF -kappa B: implications for differential gene expression in T helper cell subsets." PROC. NATL. ACAD. SCI. USA, vol. 92, December 1995, pages 11623-11627, XP002045455 see the whole document ---	1-21, 30-64, 67-87
A	TARA, DAVID ET AL: "Characterization of the constitutive and inducible components of a T cell IL - 4 activation responsive element" J. IMMUNOL. (1995), 154(9), 4592-602 CODEN: JOIMA3;ISSN: 0022-1767, XP002045456 see the whole document ---	
Y	WIERENGA E A ET AL: "Reduced NF -AT binding at the IL - 4 -CLE0. element in TH2 cells of atopic patients." 9TH INTERNATIONAL CONGRESS OF IMMUNOLOGY. THE 9TH INTERNATIONAL CONGRESS OF IMMUNOLOGY;MEETING SPONSORED BY THE AMERICAN ASSOCIATION OF IMMUNOLOGISTS AND THE INTERNATIONAL UNION OF IMMUNOLOGICAL SOCIETIES, SAN FRANCISCO, CALIFORNIA, USA, JULY 23-29,, XP002045458 see the whole document ---	1-21, 67-87
P,X	HODGE M R ET AL: "NF-AT-driven interleukin-4 transcription potentiated by NIP45." SCIENCE (WASHINGTON D C) 274 (5294). 1996. 1903-1905. ISSN: 0036-8075, 13 December 1996, XP002045448 see the whole document ---	1-64, 67-87

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	HO I-C ET AL: "The proto-oncogene c - maf is responsible for tissue-specific expression of interleukin-4." CELL 85 (7). 1996. 973-983. ISSN: 0092-8674, 28 June 1996, XP002045459 see the whole document ---	1-64, 67-87
P,X	PELTZ, GARY: "Transcription factors in immune-mediated disease" CURR. OPIN. BIOTECHNOL. (1997), 8(4), 467-473 CODEN: CUOBE3;ISSN: 0958-1669, XP002045460 see page 470, column 2; figure 2 ---	1-21, 30-64, 67-87
P,Y	FRANCASTEL C ET AL: "c-Jun inhibits NF-E2 transcriptional activity in association with p18/maf in Friend erythroleukemia cells." ONCOGENE, vol. 14, 1997, pages 873-877, XP002045461 see the whole document ---	1-64, 67-87
P,X	CASOLARO V ET AL: "Molecular mechanisms of interleukin-4 production" INTERNATIONAL JOURNAL OF IMMUNOPATHOLOGY, vol. 10, no. 2, Supplement, 1997, pages 1-5, XP002045467 see the whole document ---	1-21, 30-64, 67-87
X	IGARASHI K ET AL: "Activity and expression of murine small Maf family protein MafK." JOURNAL OF BIOLOGICAL CHEMISTRY 270 (13). 1995. 7615-7624. ISSN: 0021-9258, XP002045548	22,25, 26,29
Y	see the whole document ---	23,24, 27,28
Y	KURSCHNER C ET AL: "The maf proto-oncogene stimulates transcription from multiple sites in a promoter that directs Purkinje neuron-specific gene expression." MOLECULAR AND CELLULAR BIOLOGY 15 (1). 1995. 246-254. ISSN: 0270-7306, XP002045549 see the whole document ---	22-29

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	K. KATAOKA ET AL.: "Small Maf proteins heterodimerize with Fos and may act as competitive repressors of the NF-E2 transcription factor." MOLECULAR AND CELLULAR BIOLOGY, vol. 15, no. 4, April 1995, pages 2180-2190, XP002055946 see the whole document ---	22-29
Y	K. KATAOKA ET AL.: "MafB, a new maf family transcription activator that can associate with Maf and Fos but not with Jun." MOLECULAR AND CELLULAR BIOLOGY, vol. 14, no. 11, November 1994, pages 7581-7591, XP002055947 see the whole document ---	22-29
Y	K. IGARASHI ET AL.: "Conditional expression of the ubiquitous transcription factor MafK induces erythroleukemia cell differentiation." PROC. NATL. ACAD. SCI. USA., vol. 92, August 1995, pages 7445-7449, XP002055948 see the whole document ---	22-29
P,Y	M.H. SIEWEKE ET AL.: "MafB represses erythroid gene and differentiation through direct interaction with c-Ets-1" LEUKEMIA , vol. 11, no. 3, April 1997, pages 486-488, XP002055949 see the whole document -----	22-29

INTERNATIONAL SEARCH REPORT	International application No. PCT/US 97/06708

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [ ] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. [X] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[X] No protest accompanied the payment of additional search fees.

INVITATION TO PAY ADDITIONAL FEES

International application No.

PCT/US 97/06708

1. Claims: 1-21, 30-64, 67-87 {completely},

Method for modulating production of a T helper type 2 {Th2}-associated cytokine comprising contacting the cell with an agent that moduclates the expression or activity of a transcription factor such that production of the Th2-associated cytokine is modulated, wherein the transcription factor cooperates with a Nuclear factor of Activated T cells {NF-AT} family protein to regulate expression of the Th2-associated cytokine,
wherein the transcription factor is a maf family protein or NIP45 and,methods for identifying a compound that modulates the activity of a transcription factor that cooperates with a NF-AT family protein to regulate expression of a Th2-associated cytokine gene, where the transcription factor is a maf family protein or NIP45. Isolated nucleic acid molecule comprising a nucleotide sequence encoding NIP45, or encoding a protein with at least 60% homology to the amino acid sequence of SEQ ID NO:6 and interacting with the Rel Homology Domain of an NF-AT family protein, or hybridizing under stringent conditions to the nucleotide sequence of SEQ ID NO:5, encoding mouse NIP45, encoding human NIP45, encoding an NIP45 fusion protein, the antisense coding strand of the nucleic acid, vectors comprising said nucleic acids and host cells incorporating said vectors, method of producing NIP45 protein comprising culturing said host cells, isolated NIP45 protein and proteins encoded by said nucleic acids, antigenic peptides of NIP45, antibodies that bind NIP45 and nonhuman transgenic animals containing a transgene encoding NIP45 protein.

2. Claims: 22-29, 65 and 66

Recombinant expression vectors comprising a nucleotide sequence encoding a maf family protein operatively linkined to regulatory sequences that direct expression of the maf family protein specifically in lymphoid or hematopoietic cells, host cells incorporating said vector and non-human transgenic animals carrying a transgene encoding a maf family protein.